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14. ABSTRACT

Neural cell differentiation is a complex set of events beginning with cells responding to both soluble and cell contact-dependent external signals. These signals activate pathways that lead to changes in gene expression patterns, which ultimately give rise to the differentiated cell phenotype. In these studies, potential mechanisms regulating different aspects of oligodendrocyte differentiation were explored. Specifically, these studies examined the contribution of gene and protein localization to the establishment and/or maintenance of terminally differentiated oligodendrocyte gene expression patterns and the role of myelin transcription factor 1 (Myt1) in the regulation of oligodendrocyte proliferation and differentiation. Myt1 is a zinc-finger DNA-binding protein that is expressed in neural progenitors and is localized to discrete domains within the nucleus of oligodendrocyte progenitors. Primary oligodendrocyte lineage cells were examined during cell differentiation in order to study the localization of the highly expressed tissue-specific proteolipid protein gene relative to nuclear proteins such as Myt1 and splicing factors within interphase nuclei. These data support a nuclear organization model in which nuclear proteins and genes exhibit specific patterns of distribution within nuclei, and activation of tissue-specific genes is associated with changes in protein distribution rather than changes in gene localization. Myt1 contains six zinc-finger DNA-binding domains with sets of two N-terminal and of four C-terminal zinc-fingers. A retroviral expression system was used to overexpress the four zinc-finger DNA-binding domain of Myt1 (4FMyt1) which lacks the putative domains for protein-protein interaction and transcriptional activation. In a dominant negative study, expression of 4FMyt1 inhibited both proliferation and differentiation of oligodendrocyte progenitors. These data indicate that Myt1 contributes to the regulation of oligodendrocyte lineage development in the transitional period between proliferating progenitor cells and terminally differentiated oligodendrocytes. These studies demonstrate the importance of Myt1 and nuclear organization to the regulation of oligodendrocyte progenitor differentiation and the establishment of tissue-specific gene expression patterns.

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Abstract

Thesis Title: Nuclear Organization and Myt1 Interaction in Transcriptional Control of Neural Cell Differentiation

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Nuclear Organization and Myt1 Interaction in Transcriptional Control of Neural Cell Differentiation

By

Joseph Allen Nielsen

Thesis submitted to the faculty of the Molecular and Cell Biology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2002

Dedication

The completion of this thesis is dedicated to my parents: to my father for his guidance, support, and intellectual stimulation, and to my mother who taught me that with optimism, patience, and perseverance anything is possible.

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I would first like to thank my mentor Dr. Regina Armstrong for providing superior guidance and every resource a graduate student needs to be successful. Dr. Armstrong is an excellent scientist and teacher with an amazing ability to organize and to manage time. She provides students with advisor accessibility beyond all reasonable expectations, and somehow with all of her responsibilities, still manages to offer helpful insight and suggestions when a question arises. In addition to all this, she is a wonderful person who genuinely cares about the welfare of everyone around her. For these reasons, I consider myself extremely fortunate to have had the opportunity to train in her laboratory.

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Introduction

During development cells respond to extracellular signals which set in motion a cascae of intracellular events leading to cell fate determination and terminal differentiation with cell type-specific gene expression patterns [1]. Gene expression is thought to be regulated at multiple levels from response element-regulatory protein interactions up to the three-dimensional organization of chromatin in the nucleus [2, 3]. However, the role of nuclear organization in regulating tissue-specific gene expression and the transcriptional control of oligodendrocyte differentiation has not been well defined.

Oligodendrocytes are the central nervous system (CNS)[1] cells that produce myelin sheaths, which surround axons and facilitate efficient nerve impulse conduction. Oligodendrocytes arise in the ventral region of the spinal cord in response to soluble factors such as sonic hedgehog secreted from the notochord and floor plate [4]. Oligodendrocytes also respond to cell contact-dependent interactions from the notch-signaling pathway. The notch receptor ligand jagged1 inhibits oligodendrocyte differentiation [5]. These external signals initiate a transcriptional cascade leading to oligodendrocyte cell fate determination and regulation of differentiation. In the mouse, oligodendrocyte specification begins between embryonic day 12 and 14 [6]. Oligodendrocytes progenitors then migrate and proliferate extensively to populate the developing CNS, and when progenitors receive the appropriate signals they differentiate into

[1] For list of abbreviations see appendix A

mature oligodendrocytes sending out multiple processes to begin myelinating axons primarily during the postnatal period of development [7].

Transcriptional Regulation

Transcriptional regulation is critical in controlling cell differentiation as tissue-specific genes are activated and other sets of genes are repressed during terminal differentiation. Transcription is regulated by both *cis* and *trans* acting elements in the context of chromatin, which plays an important role in transcriptional regulation [2]. Transcriptional activation requires the preinitiation complex, which consists of RNA polymerase II and a large complex of over 50 general transcription factors. The general transcription factors include TFIID which is a multi-subunit complex consisting of the TATA binding protein that recognizes the promoter and at least 10 additional subunits. In addition to the general transcription factors, transcriptional activation requires activators that bind to enhancer sequences usually found upstream of the promoter. Activators consist of a DNA-binding domain, which recognizes a sequence-specific response element, and an activation domain that mediates the effect on transcription. Several classes of activation domains have been characterized, and a common feature of many activation domains is a concentration of acidic amino acids. For example, the activation domain of hepatocyte nuclear factor 4 contains acidic residues which act in combination with hydrophobic aromatic residues [8]. Coactivators are thought to mediate the interaction between activators and the preinitiation complex. Several models have been proposed to

explain the positive effect of activators on transcription [2]. Activator interactions may bring chromatin remodeling factors to the promoter, facilitate the recruitment of the preinitiation complex to the promoter, increase the stability of the preintiation complex, or promote a rate-limiting step in the assembly of the preinitiation complex. The net effect of the activator interactions with the preinitiation complex is an increase in the rate of initiation of RNA polymerase transcription.

Gene regulation takes place within the constraints of chromatin. Chromatin consists of repeating units of approximately 200 bp of DNA wrapped around a core of eight histone proteins. The DNA-histone complex is folded into higher order structures to further compact chromatin. Chromatin has a default repressive effect on transcription by preventing access of transcription factors to promoters and response elements [9]. Proteins that alter chromatin structure play an important role in transcriptional regulation, and can be divided into chromatin modifying proteins and chromatin remodeling proteins [10]. Chromatin remodeling proteins such as the SWI2/SNF2 family can alter chromatin conformation or shift the position of nucleosomes in the chromatin with the hydrolysis of ATP. These changes in chromatin structure can have either a positive or a negative effect on transcription [11].

A second class of chromatin modifying proteins adds chemical groups such as acetyl and methyl groups to histones or other regulatory proteins [12]. Acetylation of lysine residues on the core histones has proven to be an important mechanism for gene regulation. For example, the thyroid hormone receptor

recruits histone acetylases and deacetylases to the promoter of the genes it regulates. In the presence of bound ligand, thyroid hormone receptor binds to the coactivator CREB binding protein (CBP) where CREB is the cAMP-response-element-binding-protein. CBP then recruits p/CAF and $TAF_{II}250$ [13] which contain histone acetylase activity, and can modify chromatin by the addition of acetyl groups to the core histones. This is thought to neutralize the positive charge on lysine residues and decrease affinity for the negatively charged DNA backbone leading to a more accessible chromatin conformation. The net effect is a decrease in the default repressive state of chromatin by allowing access to the promoter and initiation of RNA polymerase transcription.

Gene transcription is initiated when the correct combination of activators, coactivators, chromatin remodeling proteins, and general transcription factors are recruited to the promoter. For each gene the order of assembly and the components can be different [14]. However, the principle of gene activation is thought to occur through the combinatorial control of cell type-specific, cell stage -specific, and ubiquitous transcription factors assembling on the promoter and enhancers.

Transcriptional repression can be mediated in a similar manner, with the recruitment of corepressors instead of coactivators. Using the thyroid hormone receptor as an example, in the absence of ligand the thyroid hormone receptor binds to the protein N-CoR which recruits mSin3 and mRPD3. This complex contains histone deacetylase activity and can mediate gene repression. [15]. In addition, neural gene repression can be mediated by the recruitment of

corepressors to genes that contain the *cis*-regulatory sequence, neural restrictive silencer [16]. The neural restrictive silencer factor can bind to genes that contain this silencer leading to the recruitment of histone deacetylases to the promoter. In these examples, histone deacetylases remove acetyl groups from positively charged lysine residues on the core nucleosome histones. This is thought to increase the electrostatic interaction between the core histones and the negatively charged DNA backbone, and lead to a more compact chromatin conformation and gene repression [9]. Additional mechanisms of gene repression are mediated by methylation of the promoter of genes that are silenced. Methyl-CpG binding proteins bind to methylated DNA, and have also been shown to bind to histone deacetylases indicating that methylation may act in combination with histone deacetylation to silence gene expression [17].

Beyond the level of the transcriptional machinery, additional levels of gene regulation involving the three-dimensional organization of chromatin and regulatory proteins in the nucleus are thought to be important. Insulators are DNA sequences that regulate the interaction of enhancers with their promoters, and prevent enhancers from acting on other targets. This is thought to partition the nucleus into transcriptionally active and repressive domains with the formation of additional levels of chromatin compaction and organization [18]. In *Drosophila*, stable gene repression can be mediated by the polycomb group of proteins. Genes containing the polycomb response element insulator bind to polycomb proteins forming a complex, which are found in discrete nuclear domains [19], and are thought to help establish higher order chromatin

organization and transcriptional repression [18]. Mammalian homologs of the polycomb family have been identified and have been implicated in the regulation of HOX gene expression [20].

Additionally, the localization of genes to regions of the nucleus containing heterochromatin may be a mechanism of silencing gene expression. Studies in yeast have reported that the localization of a gene to the nuclear periphery is associated with transcriptional repression of the yeast mating type gene [21]. The silent information regulator family of proteins is thought to help mediate repression by nucleating the condensation of chromatin at the nuclear periphery. The nuclear periphery is known to contain heterochromatin in many cell types including oligodendrocytes, which have regions of heterochromatin as well as areas of euchromatin at the nuclear periphery [22]. However, active genes have been reported to preferentially distribute to either the nuclear periphery or the nuclear interior indicating that the nuclear periphery may not be an entirely transcriptionally repressive environment [23, 24]. These studies indicate that the position of a gene within the three-dimensional space of the nucleus may be an important regulatory mechanism, and the organization of chromatin may be important for the establishment of gene expression patterns in terminally differentiated cells.

In addition to the contributions of chromatin organization to gene regulation, there is evidence that the organization of nuclear proteins may also be important for gene regulation. For example, acute promyelocytic leukemia (APL) is caused by a translocation event that fuses the promyelocytic protein

(PML) gene to the retinoic acid receptor α gene. This produces a fusion protein that correlates with the transformation of myeloid lineage cells [25]. PML is normally found localized to discrete nuclear compartments, but in APL transformed cells the localization of PML is disrupted [26]. Treatment of APL cells with retinoic acid restores the normal nuclear localization of PML, and eliminates the transformed phenotype indicating that the proper nuclear localization of PML may be critical for its normal function [27]. The function of PML and the PML nuclear compartments is unknown, but PML colocalizes with the transcriptional coactivator CBP, indicating a potential role in transcriptional regulation that may be important in controlling cell differentiation [28].

Many transcription factors are also found localized to nuclear domains. Bromouridine-triphosphate labeling of nascent RNA transcripts has shown only partial overlap with transcription factor domains, indicating that not all transcription factor compartments are active sites of transcription [29]. In addition, several studies have failed to find an association of transcription factor domains with the genes they regulate indicating that transcription factor domains may serve as storage sites [30, 31]. However, nuclear structures called coiled bodies or Cajal bodies contain a number of proteins including the proximal element sequence-binding transcription factor, which regulates snRNA gene transcription. Often Cajal bodies are found adjacent to snRNA genes, indicating that coiled bodies may serve to provide transcription factors to sites of snRNA transcription [32].

Gene regulation also occurs post-transcriptionally in processes such as RNA splicing. Many splicing factors are found concentrated in discrete nuclear domains, referred to as splicing factor compartments (SFCs) [3]. Numerous studies have identified transcriptionally active genes associated with the periphery of SFCs [33, 34], and SFCs are thought to serve as storage sites from which splicing factors are recruited to adjacent transcriptionally active genes [33].

Recent studies have shown that RNA splicing can be coupled with gene transcription by protein-protein interactions. Many splicing factors contain a sequence enriched in arginine and serine residues called the RS domain. The RS domain was shown to be critical for the function of the splicing factor (Su(w ^{a})). When the RS domain was deleted, (Su(w ^{a})) no longer localized to SFCs and was unable to splice efficiently [35]. Additionally, the C-terminal domain of RNA polymerase II was found to be essential for the recruitment of splicing factors to sites of transcription [36]. The sequestration of splicing factors into SFCs may play a role in alternative splice site selection [3]. Different members of the RS family of splicing factors show preference for splice site selection of E1a transcripts [37]. In addition, the splicing factor SC35 was recently shown to be important in the splice site selection of the CD45 gene in an SC35 null mouse leading to a defect in T-cell maturation [38]. These studies indicate a potential mechanism to regulate developmental changes in splice site selection by differential regulation of splicing factor availability in the nucleus.

Transcriptional Regulation of Oligodendrocyte Differentiation

Regulation of gene expression is critical for cell differentiation, however the transcriptional control of oligodendrocyte differentiation has not been well characterized. There is a relatively short list of approximately 20 transcription factors whose expression pattern indicates they might play a role in regulating oligodendrocyte cell fate determination and differentiation [39]. Sonic hedgehog induces the expression of the basic helix-loop-helix transcription factors Olig1 and Olig2, two of the earliest transcription factors expressed in oligodendrocytes [6]. Recent knockout studies demonstrated the importance of Olig2 for oligodendrocyte development showing a complete absence of differentiated oligodendrocytes in the spinal cord of Olig2 null mice [40]. In addition, the transcription factor Nkx2.2 in combination with Olig2 can promote the ectopic formation of oligodendrocytes in the chick spinal cord [41]. Additional transcription factors expressed in oligodendrocytes include members of the nuclear hormone receptor family including the thyroid hormone receptor, peroxisomal proliferator-activated receptor, and the retinoic acid receptor. Other transcription factor families that are expressed in oligodendrocytes include members of the POU-homeodomain family, SOX family members, and zinc-finger containing transcription factors [39]. Consensus binding sites have been defined for many of these factors and many myelin gene promoters contain binding sites for these factors indicating a potential a role in regulating myelin gene expression [39]. However, there is little functional data to determine how these factors might be regulating oligodendrocyte differentiation.

Myelin Transcription factor 1 (Myt1) is a zinc-finger DNA-binding protein that was cloned in an expression screen for proteins that bind to a sequence in the proteolipid protein (*PLP*) gene promoter [42]. PLP is a myelin structural protein that is important for myelin sheath compaction, and is highly expressed in differentiated oligodendrocytes. Myt1 consists of six zinc-finger domains with the cysteine-cysteine-histidine-cysteine zinc coordination motif. In this motif, the three cysteines and one histidine residue coordinate a single zinc atom, which stabilizes the secondary structure. The zinc-fingers are arranged with two N-terminal fingers and four C-terminal fingers, and both sets of fingers can bind independently to a site in the *PLP* promoter [42]. Myt1 contains a putative acidic transcriptional activation domain, and an alpha-helical protein-protein interaction domain (Figure 1). Myt1 also contains multiple protein kinase C and calmodulin dependent kinase II consensus phosphorylation sites [42]. A Myt1 consensus DNA binding site has been defined and consists of a core TGG site. The Myt1 consensus site can be found in the *PLP* promoter in several different locations [43]. In addition, the widely separated sets of zinc-fingers indicate that Myt1 may bind to multiple sites in the promoter of genes it regulates.

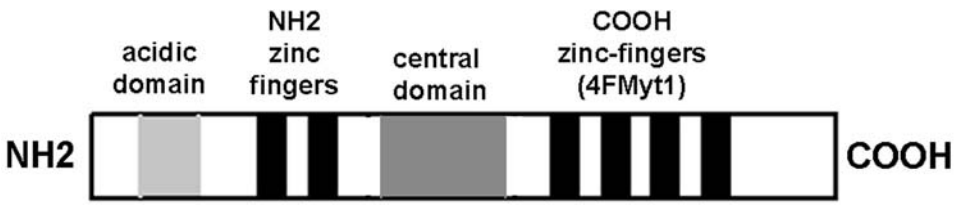

Figure 1: Schematic of myelin transcription factor 1. Zinc-fingers are shown in black, the acidic putative transcriptional activation domain in light gray, and the central protein-protein interaction domain in dark grey.

In the developing CNS, Myt1 is expressed in the neuroepithelial germinal zones of the spinal cord and brain, and is expressed in both neuronal and oligodendrocyte progenitors [44, 45]. Myt1 is expressed in the earliest cells that can be identified as oligodendrocytes, and continues to be expressed as oligodendrocytes begin to differentiate [44]. Myt1 expression is down-regulated as oligodendrocytes terminally differentiate and *PLP* accumulates [44]. In the mouse, Myt1 expression begins at approximately embryonic day 12, around the time when the first oligodendrocytes begin to appear in the ventral spinal cord [7, 45]. Myt1 expression peaks at about embryonic day 15 and then begins to decline into the postnatal period [45]. There remains a population of cells that continue to express Myt1 in the adult brain germinal zones [46], which may provide a source of progenitors for injury and repair [47, 48]. In addition, Myt1 expression is up-regulated in a spinal cord injury model [49]. Myt1 is also up-regulated in gliomas, and increased Myt1 expression correlated with higher grades of gliomas [46]. These expression studies demonstrate that the developmental and pathological expression patterns of Myt1 strongly correlate with the proliferative immature cell types within the oligodendrocyte lineage.

Myt1 is the prototypic member of a family of zinc-finger proteins with the cysteine-cysteine-histidine-cysteine zinc-finger DNA-binding motif. A second member of the family called Myt1-like contains a similar primary structure [45]. However, the Myt1-like expression pattern is different, being only expressed in a subset of postmitotic neurons. A third member of the family called neural zinc finger-3 has been described with a similar structure although lacking the acidic domain [50]. The zinc-finger domains within the Myt1 family are highly conserved with 70-90% identity at the amino acid level [45]. There is also a high degree of conservation between the species human, rat, mouse, and *Xenopus* versions of Myt1, which all show a high degree of sequence homology within the zinc fingers. Taken together, the expression pattern of this protein family indicates a potential role in regulating neural cell development. Consistent with these mammalian Myt1 expression data, a functional study with a *Xenopus* version of Myt1 was shown to promote the differentiation of neurons [51].

Overview of Thesis Studies

We used a primary oligodendrocyte culture system to study transcriptional control of oligodendrocyte differentiation at both the nuclear organization and protein level. Previous work demonstrated that Myt1 was distributed in a non-homogenous nuclear pattern [44]. The partitioning of Myt1 into nuclear domains indicated that Myt1 function may depend on higher order nuclear organization for its function. Evidence in the literature of a three-dimensional component to gene regulation prompted the examination of the contribution of gene and protein

localization to the establishment and/or maintenance of terminally differentiated oligodendrocyte gene expression patterns. Using genomic in situ hybridization to detect the *PLP* and the myelin basic protein (*MBP*) genes, and immunostaining to detect nuclear proteins, we examined different aspects of nuclear organization and its contribution to myelin-specific gene expression during oligodendrocyte differentiation. In addition to the striking nuclear distribution, the temporal and spatial developmental expression patterns of Myt1 indicated that Myt1 might act as a pro-differentiation factor regulating oligodendrocyte differentiation. The primary structure of Myt1 also indicated a possible transcriptional regulatory function. Therefore, using a retroviral expression system we examined the function of Myt1 in developing oligodendrocytes.

Hypothesis: Nuclear organization and Myt1 are important in the regulation of oligodendrocyte specific gene expression and differentiation.

Aim 1: Determine the contribution of nuclear organization to oligodendrocyte specific gene expression.

Aim 2: Determine the role of Myt1 in developing oligodendrocytes.

These studies were expected to help elucidate the mechanisms of tissue-specific and coordinate gene expression during oligodendrocyte differentiation, and to determine the function of Myt1 in the regulation of oligodendrocyte development. A basic understanding of the mechanisms that regulate oligodendrocyte proliferation and differentiation are important initial steps toward any future

therapeutic approaches that could be used for the treatment of human diseases involving oligodendrocytes, such as multiple sclerosis.

Paper 1

Nuclear Organization in Differentiating Oligodendrocytes

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Key words: gene expression, nuclear organization, oligodendrocyte, proteolipid protein, splicing factors.

Abstract

Many studies have suggested the three-dimensional organization of chromatin and proteins within the nucleus contributes to the regulation of gene expression. The current study tests multiple aspects of this nuclear organization model within a primary cell culture system. Oligodendrocyte lineage cells were examined to facilitate analysis of nuclear organization relative to a highly expressed tissue-specific gene, proteolipid protein (*PLP*), which exhibits transcriptional up-regulation during differentiation from the immature progenitor stage to the mature oligodendrocyte stage. Oligodendrocyte lineage cells were isolated from brains of neonatal male rodents, and differentiation from oligodendrocyte progenitors to mature oligodendrocytes was controlled with culture conditions. Genomic in situ hybridization was used to detect the single copy of the X-linked *PLP* gene within each interphase nucleus. The *PLP* gene was not randomly distributed within the nucleus, but was consistently associated with the nuclear periphery in both progenitors and differentiated oligodendrocytes. *PLP* and a second simultaneously up-regulated gene, the myelin basic protein (*MBP*) gene, were spatially separated in both progenitors and differentiated oligodendrocytes. Increased transcriptional activity of the *PLP* gene in differentiated oligodendrocytes corresponded with local accumulation of SC35 splicing factors. Differentiation did not alter the frequency of association of the *PLP* gene with domains of myelin transcription factor 1 (Myt1), which binds the *PLP* promoter. In addition to our specific findings related to the *PLP* gene, these data obtained from primary oligodendrocyte lineage cells support a nuclear organization model

in which 1) nuclear proteins and genes can exhibit specific patterns of distribution within nuclei and, 2) activation of tissue-specific genes is associated with changes in local protein distribution rather than spatial clustering of coordinately regulated genes. This nuclear organization may be critical for complex nucleic acid-protein interactions controlling normal cell development, and may be an important factor in aberrant regulation of cell differentiation and gene expression in transformed cells.

Introduction

Within the nucleus, DNA replication and transcription as well as RNA splicing each require coordination among many different proteins interacting with DNA and RNA. Organizing principles within the nucleus have been proposed to facilitate these complex nuclear functions (Lamond and Earnshaw, 1998; Misteli, 2000). Chromosomes, genes, RNA transcripts, and proteins each localize to discrete yet dynamic domains that may reflect spatial compartmentalization to facilitate nuclear functions. Among the multitude of detectable nuclear domains, it is now important to identify spatial and temporal relationships that have functional significance.

The localization of a gene within the nucleus may be an important regulatory mechanism. For example, targeting of genes to regions of the nucleus containing heterochromatin may be one mechanism of silencing gene expression (Brown et al., 1999). Although the peripheral region of the nucleus is known to contain heterochromatin in many cell types, active genes may preferentially distribute to either peripheral or central regions (Marshall et al., 1996; Croft et al., 1999).

Many nuclear proteins are found concentrated in discrete domains (Lamond and Earnshaw, 1998). Numerous studies have identified transcriptionally active genes associated with the periphery of nulcear domains enriched in splicing factors, called splicing factor compartments (SFCs) (Misteli et al., 1997; Smith et al., 1999; Dundr and Misteli, 2001). Additionally, [^{3}H]uridine and Br-UTP incorporation into nascent RNA transcripts labels the periphery of

SFCs indicating that this region is a site of active transcription (Misteli and Spector, 1998; Wei et al., 1999). The periphery of SFCs is also enriched in hyperacetylated chromatin, which is considered a marker for the transcriptionally active state of chromatin (Hendzel et al., 1998). SFCs may serve as storage sites from which splicing factors are recruited to adjacent transcriptionally active genes (Misteli et al., 1997). Many transcription factors are also concentrated into domains throughout the nucleus, and an unresolved question is whether these sites represent active sites of transcription, storage sites, or other undefined functional accumulations (Elefanty et al., 1996; Grande et al., 1997; Jolly et al., 1997; Schul et al., 1998).

The organization of both nuclear proteins and chromatin changes during cell differentiation (Antoniou et al., 1993; Santama et al., 1996). In this study, we sought to identify changes in nuclear organization occurring during cell differentiation that might contribute to the establishment of terminally differentiated gene expression patterns. Transformed cell lines have been used extensively to study nuclear organization, but established cell lines often have altered differentiation characteristics and may not accurately reflect regulation of tissue-specific gene expression. Therefore, it is important to test relevant nuclear distributions in the context of tissue-specific genes that are activated during differentiation of primary cells. In this study, we used a primary culture system with specific advantages for analysis of nuclear organization relative to cell differentiation. Oligodendrocytes are central nervous system (CNS) cells that produce myelin sheaths, which surround axons to facilitate efficient nerve

impulse conduction. During differentiation of oligodendrocytes, there is a simultaneous up-regulation of a set of tissue-specific genes that encode the proteins required for synthesis of the myelin sheath. These tissue-specific genes must be appropriately regulated for normal myelination during CNS development and for remyelination associated with CNS regeneration.

This experimental system has several advantages for studying changes in nuclear organization during cell differentiation: 1) primary oligodendrocyte cultures mimic the in vivo progression of differentiation and expression of myelin-specific proteins (Dubois-Dalcq et al., 1986), 2) oligodendrocyte up-regulation of transcription of the proteolipid protein (*PLP*) gene during differentiation can be controlled by manipulating the culture conditions, 3) cells isolated from male animals have a single active allele of the X-linked *PLP* gene, 4) a second myelin-specific gene, myelin basic protein (*MBP*), is transcriptionally up-regulated at approximately the same time as *PLP* both in vivo and in vitro.

In this primary culture model system, we used genomic in situ hybridization to monitor the nuclear localization of the *PLP* and *MBP* myelin-specific genes relative to differentiation and transcriptional activation within interphase oligodendrocyte nuclei. In addition, genomic in situ hybridization was combined with immunostaining for the splicing factor SC35 (Fu and Maniatis, 1990) and the DNA-binding protein myelin transcription factor 1 (Myt1) (Kim and Hudson, 1992) to determine the spatial relationship between myelin-specific genes and related nuclear proteins as the cells undergo terminal differentiation.

Materials and Methods

Cell culture

Primary cultures from male neonatal rat brains were prepared as previously described (Armstrong, 1998). Briefly, postnatal day 2 rat brains were dissociated, plated in tissue culture flasks, and allowed to grow for 7-10 days. The flasks were placed on a rotary shaker to dislodge immature oligodendrocyte lineage cells, which were then plated onto poly-D-lysine coated chamber slides. Progressive stages of differentiation within the oligodendrocyte lineage can be identified with cell type-specific markers and based upon the characteristic morphology of each stage (Armstrong, 1998). To obtain cultures of immature oligodendrocyte lineage phenotypes, cells were grown in medium containing 10 ng/ml of PDGF-AA and 10 ng/ml FGF2 (R and D Systems, Minneapolis, MN)(Armstrong, 1998). Pre-oligodendrocyte progenitors were obtained by allowing these cultures to adhere for only 2 hours before fixation. The majority of plated cells progressed to oligodendrocyte progenitors if allowed to grow, and with PDGF and FGF treatment these cells remained progenitors until fixation at day 3. Differentiated oligodendrocytes were obtained by plating in medium without PDGF and FGF and allowing the cells to mature during 3 days in culture. Astrocytes served as a glial cell control that is not part of the oligodendrocyte lineage. Astrocytes were obtained from the same primary rat brain glial cultures by purification of the population that remained adhered to the initial flasks after the oligodendrocyte lineage cells were dislodged. Primary mouse

oligodendrocytes were prepared in a manner similar to the rat oligodendrocyte lineage cells. In experiments to inhibit RNA polymerase II transcription, cells were treated with 5 μg/ml α-amanitin (Roche Applied Science, Indianapolis, IN) for 2 hours prior to fixation. All animals were handled in accordance with procedures approved by the USUHS Institutional Animal Care and Use Committee. All quantitation was based on data combined from at least 3 independent preparations of cells from separate litters of animals.

PLP mRNA in situ hybridization

In situ hybridization for *PLP* mRNA was performed as previously described (Redwine and Armstrong, 1998). Briefly, cells were fixed with 4% paraformaldehyde, acetylated, and prehybridized with RNA hybridization buffer (DAKO, Carpenteria, CA). A 980 bp cDNA corresponding to the entire coding region of the mouse *PLP* gene, derived from pLH116 (Hudson et al., 1987), served as a template to incorporate digoxigenin-11-UTP (Roche Applied Science, Indianapolis, IN) using in vitro transcription (Ambion, Austin, Texas). The probe was denatured and allowed to hybridize overnight. The probe was detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Applied Science, Indianapolis, IN) followed by NBT/BCIP colorimetric detection (DAKO, Carpenteria, CA).

Genomic in situ hybridization

Cells were fixed with 2% paraformaldehyde and processed using a modified protocol for genomic in situ hybridization detection (Johnson et al., 1991). The cells were extracted with 0.5% NP40 detergent and dehydrated through graded ethanols. The cells were pre-treated with hybridization buffer without probe. The target DNA and probe, labeled with digoxigenin-11-dUTP using nick translation, were denatured and then hybridized overnight. The *PLP* genomic in situ hybridization probe was generated from a 3.7 kb fragment of the rat *PLP* promoter (Cambi and Kamholz, 1994). Detection of digoxigenin labeled probe was performed using a tyramide signal amplification system™ (NEN, Boston, MA). Probes were detected with biotinylated anti-digoxin antibody (Jackson ImmunoResearch, West Grove, PA) followed by steptavidin horseradish peroxidase (HRP). HRP was then used to catalyze the deposition of tyramide-FITC at the site of probe binding. The specificity of the hybridization was confirmed by absence of signal using the following conditions 1) no probe, 2) probe and target not denatured, 3) hybridization competition with 100-fold excess of non-labeled probe.

For the double genomic hybridization experiments in mouse oligodendrocyte cultures, a mouse *PLP* probe corresponding to 4.0 kb of the mouse *PLP* promoter (isolated from an EcoRI and PstI digest of pMu*PLP*9; unpublished, L.D.H.) was labeled with FITC-11-dUTP (Roche Applied Science, Indianapolis, IN). A mouse *MBP* probe corresponding to 3.0 kb of the mouse *MBP* promoter (isolated from a XbaI and SalI digest of JCC137; unpublished,

L.D.H.) was labeled with digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN). The probes were hybridized overnight simultaneously, and then detected sequentially using a tyramide signal amplification system. The *PLP* probe was detected with anti-FITC conjugated with HRP followed by tyramide-dinitrophenyl, anti-dinitrophenyl conjugated with HRP, and then tyramide-FITC. The peroxidase activity was quenched with a 15 minute 0.02 M HCL treatment, and the digoxigenin-*MBP* probe was detected with anti-digoxin conjugated with biotin, followed by streptavidin-HRP, and tyramide-Cy3. The specificity of each hybridization and detection scheme was confirmed by absence of signal in hybridizations using each single probe followed by combined anti-FITC and anti-digoxin detection protocols. We also confirmed our ability to inactivate the HRP, as required to quench the *PLP* detection prior to *MBP* detection. In experiments using the single *PLP* hybridization protocol, after the incubation with anti-FITC conjugated with HRP, the HRP was inactivated with 0.02M HCL and the absence of signal was confirmed following the detection protocol.

Immunostaining of nuclear proteins

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X 100. Mouse anti-SC35 monoclonal antibody (ATCC, Manassas, VA) and rabbit anti-Myt1-His polyclonal antibody (Armstrong et al., 1995) were added to slides, and incubated overnight. Following a blocking step with 5% normal donkey serum, the primary antibodies were detected with donkey anti-mouse

FITC and donkey anti-rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA). For the in situ hybridization in combination with immunostaining experiments, a 5-minute post-fixation with 4% paraformaldehyde was included before the addition of the primary antibody, and the immunostaining was then carried out as described above.

Image analysis

Two-dimensional images were collected with an Olympus IX70 epifluorescence microscope equipped with a 40x objective using a Spot2 digital camera. 3D images were collected in 0.25 μm sections through the Z-dimension with a 63x objective (1.4 NA) on a Ziess Axiophot epifluorescence microscope using a Sensicam digital camera. The haze was removed from 3D images using the deconvolve algorithm and point spread functions generated for the red and green channels within Slidebook (Intelligent Imaging Innovations, Denver, CO). The images were analyzed using Metamorph software (Universal Imaging Corporation, West Chester, PA) and domains were scored as co-localized when there was pixel overlap in the red and green channels. A micrometer was used to calculate XY resolution with one pixel = 0.18 μm at 40x. Figures were assembled in Adobe Photoshop (Adobe, San Jose, California). As a control for image registration, fiduciary markers that fluoresce in both the red and green wavelengths were imaged. In experiments where images were collected of a single sub-resolution bead (0.17 μm diameter) in both channels, the merged image had complete overlap of the red and green pixels.

Results

The tissue-specific *PLP* gene is transcriptionally up-regulated during differentiation of oligodendrocytes in primary cultures.

Transcription of the PLP gene increases developmentally as oligodendrocytes differentiate and form myelin (Macklin et al., 1991; Shiota et al., 1991). In our culture system, we demonstrate the difference in PLP gene expression in progenitors versus differentiated oligodendrocytes. Oligodendrocyte progenitors were isolated from postnatal day 2 male rat pups, and differentiation was inhibited by addition of platelet derived growth factor-AA (PDGF) and fibroblast growth factor 2 (FGF). The progenitors exhibited a characteristic bipolar morphology (Figure 1A). Only background signal was detected by in situ hybridization for *PLP* mRNA at this progenitor stage (Figure 1B). Parallel cultures grown without the addition of PDGF and FGF terminally differentiated into mature oligodendrocytes. After three days in medium without PDGF and FGF, there were high levels of *PLP* mRNA transcripts detected in cells with the characteristic highly branched morphology of differentiated oligodendrocytes (Figures 1C, D). *PLP* mRNA in situ hybridization was also performed on astrocyte cultures as a glial cell control that does not express detectable levels of *PLP* mRNA (Figures 1E, F). These data confirm our ability to control oligodendrocyte differentiation and the associated up-regulation of myelin-specific gene expression.

Active *PLP* transcription induces adjacent splicing factor compartments. The spatial relationship between the *PLP* gene and SFCs was examined as cells differentiated and up-regulated transcription from the *PLP* gene locus. The splicing required for processing the *PLP* gene is typical of mammalian genes; the *PLP* gene encompasses approximately 17kb of DNA with 7 exons (Macklin, 1992; Lewin, 1994). Oligodendrocyte lineage cell cultures and astrocyte cultures were prepared, as described in the methods. Genomic in situ hybridization with a probe directed against the promoter and upstream regulatory regions of the *PLP* gene was used to detect a single site corresponding to the X-linked *PLP* gene in a given nucleus. This genomic in situ hybridization was combined with immunofluorescence to simultaneously detect the splicing factor SC35 which labels SFCs (Figure 2). Astrocytes, which do not express detectable *PLP* mRNA (Figure 1F), exhibited co-localization of the *PLP* gene with a discrete SC35 domain in 15 $\pm$ 3% of the cells examined (Figures 2A, 3). Progenitor cells, which under these culture conditions are not expressing marked levels of *PLP* mRNA (Figure 1B), exhibited co-localization of the *PLP* gene with SC35 in 22 $\pm$ 9% of the cells (Figures 2B, 3). In contrast, in differentiated oligodendrocytes in which the *PLP* gene should be highly expressed (Figure 1D), the *PLP* gene was co-localized with SC35 in 63 $\pm$ 5% of the cells (Figures 2C, 3).

The spatial relationship between the *PLP* gene and SFCs was confirmed in the Z-axis (Figure 4). Digital optical sections were used to generate 3D reconstructions for 10% of each oligodendrocyte lineage population sampled in

the 2D analysis. A similar spatial relationship and frequency of association was found between the *PLP* gene and SC35 in this 3D analysis as with the 2D analysis.

As an additional control, the spatial relationship of SFCs was examined relative to a gene that is not expressed in mature oligodendrocytes. The interphotoreceptor retinoid binding protein (*IRBP*) gene was selected since *IRBP* is expressed by photoreceptor cells and the pineal gland (van Veen et al., 1986), but is not expressed in oligodendrocytes (D. Borst, personal communication). The *IRBP* gene co-localized with an SC35 domain in 26 $\pm$ 4% of the oligodendrocytes examined (Figures 2E, 3).

The increased frequency of association of the *PLP* gene with SFCs in mature cells was dependent on transcriptional activity. Differentiated oligodendrocytes were treated for 2 hours with 5 μg/ml α-amanitin to inhibit RNA polymerase II activity. In the presence of α-amanitin, the frequency that the *PLP* gene was associated with an SC35 domain was significantly decreased (Figures 2D, 3). This finding suggests that transcriptional activity of the *PLP* gene is required to induce adjacent SC35 accumulation.

These data demonstrate that in differentiated oligodendrocytes the *PLP* gene is frequently associated with nuclear compartments containing the splicing factor SC35. This association is not simply the result of cell differentiation, but is dependent upon *PLP* transcriptional activity.

The *PLP* gene does not exhibit radial translocation during oligodendrocyte differentiation.

The location of genes could be related to the partitioning of heterochromatin within the nucleus and might serve as a mechanism of transcriptional control. In our experiments, the *PLP* gene appeared to more typically localize within a peripheral region of the nucleus (see examples in Figures 2, 4) whereas the *IRBP* gene alleles, which are both inactive in these cells, did not have a notable nuclear localization (see example in Figure 2). This apparent differential distribution was substantiated using phase contrast microscopy to image individual nuclei combined with fluorescence imaging of the genomic in situ hybridization signal for each gene (Figure 5A). A gene was classified as within the peripheral region of the nucleus if the measured distance between the center of the in situ hybridization signal and the edge of the nucleus was less than 1.5 μm. Since the *IRBP* gene has two alleles, the cells were also scored by the relative location of both alleles as a set in each cell, which demonstrated that at least one allele was located in the central region in the majority of the cells examined (Figure 5B). This difference in localization between the *PLP* gene and the *IRBP* gene supported the interpretation that the *PLP* gene may be non-randomly localized within the nuclear periphery. The *PLP* gene location was more carefully examined using DAPI nuclear stain to identify the nuclear volume (Figure 6). Based upon preliminary measurements of the nuclear volume with imaging of DAPI fluorescence, a 1.5 μm border inside the nuclear periphery comprised approximately 50% of the nuclear area (data not shown). In

differentiated oligodendrocytes, as well as progenitors and astrocytes, the *PLP* gene was localized within this peripheral border in approximately 75% of the cells examined for each cell type (Figure 5C). When compared to a random distribution, using the calculated average area of the nucleus, in each cell type the *PLP* gene was found non-randomly associated with the peripheral region of the nucleus. The similar preferential localization in oligodendrocytes and astrocytes indicates that this peripheral localization does not correlate with transcriptional status of the PLP gene. Accordingly, the *PLP* gene does not undergo a large-scale change in radial position as progenitors differentiate into mature oligodendrocytes and up-regulate transcription from the *PLP* locus.

The transcription factor Myt1 localizes to different nuclear domains from splicing factors.

Splicing can occur as a co-transcriptional event (Misteli and Spector, 1999). Therefore, nuclear proteins involved in splicing and transcription may exhibit specific relative nuclear distributions that facilitate availability at sites of ongoing transcription and splicing. Two-color immunofluorescence was used to detect the nuclear distribution of a representative splicing factor, SC35, and a representative DNA-binding protein Myt1. Myt1 was used for this example because Myt1 binds to the *PLP* promoter and Myt1 is distributed in discrete nuclear domains in oligodendrocyte lineage cells (Armstrong et al., 1995). SC35 and Myt1 immunoreactivities exhibited very different nuclear patterns (Figures 7A, B). As previously described in other systems (Fu and Maniatis, 1990), SC35

was found in a pattern characteristic of SFCs. Myt1 immunoreactivity appeared as more numerous punctate domains scattered throughout the nucleus. Myt1 immunoreactivity was predominately excluded from the interior regions of SC35 domains, but frequently was observed in discrete accumulations associated with the periphery of SC35 domains.

The nuclear distribution of Myt1 DNA-binding protein is independent of *PLP* promoter activity.

Accumulations of DNA-binding proteins near their genomic targets may contribute to the regulation of relative interactions. Given our independent observations of *PLP* gene localization and Myt1 domains each being associated with the periphery of SFCs, it was important to examine whether the *PLP* gene and Myt1 domains were co-localized. *PLP* genomic in situ hybridization was combined with Myt1 immunostaining to determine the spatial relationship between Myt1 nuclear domains and the *PLP* gene. We analyzed three progressive stages of differentiation within the oligodendrocyte lineage: pre-oligodendrocyte progenitors, oligodendrocyte progenitors, and differentiated oligodendrocytes (Figures 8, 9). At each stage in the lineage, Myt1 immunoreactivity was associated with the *PLP* gene in approximately 50% of the cells observed. The *IRBP* gene, which is not expressed in oligodendrocytes (D. Borst, personal communication), also exhibited a similar approximately 50% frequency of association with Myt1. These data indicate that while discrete domains of Myt1 DNA-binding protein are present in nuclei, these domains do

not preferentially accumulate to detectable levels near presumptive gene targets, such as the *PLP* gene, when these targets are transcriptionally active.

Two coordinately regulated myelin genes remain spatially separated during oligodendrocyte differentiation.

To examine whether gene localization may contribute to coordinate transcriptional regulation, double genomic in situ hybridization was performed to compare the relative nuclear localization of two myelin-specific genes during oligodendrocyte differentiation. The *PLP* and the *MBP* genes were chosen for analysis because expression of each gene is up-regulated at a very similar time during oligodendrocyte differentiation. There was no co-localization of the *PLP* gene with either of the *MBP* alleles in almost every cell examined (Figure 10). The same lack of co-localization was observed in mature oligodendrocytes (31 of 31 cells), progenitors (53 of 53 cells), and astrocytes/microglia (28 of 29). In most cells, the *PLP* gene and the *MBP* gene were found in disparate regions of the nucleus. The autosomal *MBP* alleles were nearly always found clearly separated from each other (Figure 10), and were evenly distributed relative to the peripheral or the central regions of nuclei (data not shown). These data argue against a hypothesis involving gene co-localization to coordinate transcription of a set of tissue-specific genes associated with terminal differentiation.

Discussion

This study extends previous work on gene expression and nuclear organization into a primary cell culture model that undergoes differentiation to analyze changes associated with the corresponding up-regulation of tissue-specific gene expression. As a model system, we focus on the *PLP* gene in the oligodendrocyte lineage. We show that *PLP* transcriptional activity is associated with localized changes in specific nuclear protein domains. However, the *PLP* gene does not undergo large-scale translocation relative to the nuclear periphery or another myelin-specific gene, *MBP*.

The position of a gene in the three-dimensional space of the nucleus may be an important transcriptional regulatory mechanism. Ribosomal genes located on different chromosomes are segregated into the nucleolus presumably to facilitate efficient transcription, modification, and assembly of ribosomal gene products (Scheer and Hock, 1999). In contrast, when expression of the immediate early gene *c-fos* is induced in NIH-3T3 cells, the two alleles are transcriptionally active but are not located adjacent to one another (Huang and Spector, 1991). Thus, different organizing principles appear to be applied to different classes of genes. Few studies have addressed the question of whether sets of tissue-specific genes that are coordinately regulated and share similar regulatory factors also exhibit regulated spatial localization within the nucleus. One example that is available for tissue-specific genes showed that immunoglobulin genes are non-randomly and differentially positioned in the nucleus in two mature B-cell lines (Parreira et al., 1997). These immunoglobulin

genes each maintained a different topography relative to each other and to peripheral versus central regions of the nuclear volume, regardless of transcriptional activity. However, several studies have reported large scale movements of genes within the nucleus, particularly between the peripheral and central regions (Palladino et al., 1993; Brown et al., 1999; Gerasimova et al., 2000).

Our data extend these findings to developmentally regulated tissue-specific genes in primary cultures of oligodendrocyte lineage cells undergoing terminal differentiation. Our data clearly show that the *PLP* gene was not spatially associated with either *MBP* allele. This result was observed in progenitors as well as after differentiation and up-regulation of *PLP* and *MBP* transcription in oligodendrocytes. In addition, we show that the *PLP* gene is consistently associated with a peripheral nuclear localization in oligodendrocyte progenitors, differentiated oligodendrocytes, and astrocytes.

The periphery of SFCs have been demonstrated to be transcriptionally active sites based upon labeling to reveal nascent RNA transcripts (Misteli and Spector, 1998; Wei et al., 1999) and identification of increased levels of acetylated chromatin (Hendzel et al., 1998). In preliminary studies, we compared the *PLP* gene localization to regions in the nucleus enriched in acetylated chromatin, but did not find a marked association of acetylated chromatin with the *PLP* gene (data not shown). Previous studies in cell lines demonstrate splicing occurring at the site of transcription (Misteli and Spector, 1999). Importantly, our analysis allowed SFCs and the *PLP* gene to be compared at multiple stages of

regulation of the *PLP* gene locus. SC35 splicing factors accumulated in discrete nuclear compartments adjacent to sites of transcriptionally active *PLP* genes in differentiated oligodendrocytes. Isoforms of the *PLP* gene have been reported to be expressed embryonically (Ikenaka et al., 1992; Timsit et al., 1992) and in oligodendrocyte progenitors (Mallon et al., 2002). However, the abundance of *PLP* mRNA transcripts in progenitors is dramatically lower than in mature oligodendrocytes (Figure 1B). In addition, astrocytes (Figure 1F) do not express *PLP* isoforms (Fuss et al., 2000). Therefore, the detectable accumulation of splicing factors adjacent to the *PLP* gene only in oligodendrocytes is likely to be related to the active production of *PLP* transcripts. Interestingly, accumulation of SC35 splicing factors relative to active genes may be a gene-specific process (Smith et al., 1999). Thus, our results characterize the *PLP* gene locus within the class of genes that demonstrate SC35 accumulation with transcriptional activity.

Many transcription factors are found in discrete nuclear domains, and an unresolved question is whether these domains correspond with regulation of target gene transcription. Our data for the DNA-binding protein Myt1 suggests that Myt1 domains are not strictly associated with a particular state of *PLP* gene activation. We have not attempted the extensive quantitative analysis of the nuclear volume occupied by the Myt1 domains to formally compare a random occurrence with the 50% association of Myt1 domains relative to the active versus inactive states of the PLP gene. However, several other studies have not found an association of transcription factor domains relative to their presumed

genomic targets (Elefanty et al., 1996; Jolly et al., 1997). Presumably, the number of transcription factor molecules required to bind to the promoter of a target gene to regulate transcription is likely to be relatively few, which may explain why transcription factor domains are not clearly associated with sites of active transcription.

Accumulations of transcription factors into domains may still be functionally important even if detectable domains are not preferentially localized adjacent to target gene transcription sites. For example, the subnuclear localization of Runx2/CBFA1/AML3 transcription factors in discrete domains appears to be critical for tissue-specific gene expression and differentiation (Choi et al., 2001). Relative to Myt1, gliomas exhibit variable subnuclear and subcellular Myt1 immunoreactivity compared to normal oligodendrocyte lineage cells (Armstrong et al., 1997). In preliminary studies (data not shown), we determined that the Myt1 nuclear pattern was distinct from that of several other nuclear proteins, including thyroid hormone receptor β1, which is known to bind to the promoters of both *PLP* and *MBP* (Bogazzi et al., 1994; Tomura et al., 1995). Therefore, Myt1 domains exhibit a specific pattern that is not likely to reflect a general pattern of accumulated transcription factors in oligodendrocyte lineage cells. In addition, the nuclear domains of Myt1 appeared larger and less abundant than domains associated with BrUTP-labeled nascent RNA transcripts (data not shown). We predict that domains of Myt1 might serve as a mechanism to sequester and thereby regulate the concentration of available protein. This concept of regulated Myt1 availability would be consistent with our previous

observation that Myt1 immunoreactivity shifts from the nucleus to the cytoplasm as mature oligodendrocytes accumulate *PLP* protein, and Myt1 is subsequently down-regulated (Armstrong et al., 1995).

Our data support a model in which discrete functional domains are regulated by localized changes in protein distribution (Carmo-Fonseca, 2002). Recent work suggests that the nucleus is an extremely dynamic environment with many nuclear proteins showing high rates of mobility throughout the nucleus (Phair and Misteli, 2000; Carmo-Fonseca, 2002). SFCs adjacent to active genes may reflect the relative accumulation of molecules required to process multiple copies of RNA transcripts as they are generated. In contrast, DNA-binding proteins may not accumulate to detectable levels near active genes because fewer molecules are likely to be required to regulate transcription from a single copy genomic DNA.

Coordinate transcriptional control of tissue-specific genes may then be accomplished through binding interactions that regulate the local concentrations of available DNA-binding proteins, which are relevant for a given set of target genes. These protein-protein and protein-nucleic acid interactions would be expected to establish an appropriate nuclear environment for regulated transcription. Future studies will be required to test this model using methods that assess these functional interactions without dramatically disrupting the balance of concentrations and spatial relationships among nuclear elements. This model has implications for understanding the regulation of cell differentiation and tissue-specific gene expression during normal development. These

regulatory mechanisms may also provide insight for pathological observations, as in the example of aberrant protein expression in tumors and dysplasia (Weis et al., 1994; Armstrong et al., 1997).

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Abbreviations: CNS-central nervous system, *IRBP*-interphotoreceptor retinoid-binding protein, *MBP*-myelin basic protein, Myt1-myelin transcription factor 1, *PLP*-proteolipid protein, splicing factor compartments-SFCs.

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Figure Legends

Figure 1: *PLP* mRNA in situ hybridization in primary rat oligodendrocyte cultures. Phase contrast image analysis shows bipolar processes that are characteristic of progenitors (A), multiple branched processes characteristic oligodendrocytes (C), and flat fibroblastic morphology of astrocytes (E). Brightfield images show that *PLP* mRNA was not detectable in cultured progenitors (B, same field as A) or astrocytes (F, same field as E). High levels of *PLP* mRNA (blue/black signal) accumulated in differentiated oligodendrocytes (D, same field as C). Scale bar = 50 μm.

Figure 2: SC35 immunostaining combined with genomic in situ hybridization. Merged images for *PLP* genomic in situ hybridization (green) combined with SC35 immunostaining (red) in astrocytes (A), progenitors (B), oligodendrocytes (C), and oligodendrocytes treated with α-amanitin (D). *IRBP* genomic in situ hybridization (green) merged with SC35 immunostaining (red) in progenitors (E). Scale bar = 10 μm.

Figure 3: Quantitation of genomic in situ hybridization combined with SC35 immunostaining. The *PLP* gene and SC35 domains were scored as co-localized when there was pixel overlap in the red and green channels (see Figure 2). SC35 domains are more frequently co-localized with the *PLP* gene in oligodendrocytes (oligos, n=110) compared to cells that do not transcribe

detectable levels of *PLP*, i.e. astrocytes (astros, n=55), progenitors (OPs, n=116), and oligodendrocytes treated with α-amanitin (oligos+AM, n=110) ($p<0.05$; Chi-square). As a control, the *IRBP* gene, which is not expressed in oligodendrocytes, had a frequency of *PLP*/SC35 co-localization similar to cells that do not express *PLP* (n=50). Error bars = standard error of the proportion.

Figure 4: 3D reconstruction of *PLP* genomic in situ hybridization merged with SC35 immunostaining in oligodendrocytes. The *PLP* gene (green) co-localizes with SC35 immunostaining (red) to appear as yellow at the periphery of nuclear domains enriched in SC35 in oligodendrocytes. Oligodendrocytes shown as a 3D reconstruction imaged in the XY plane (A) and rotated 90 degrees to the XZ view (B). In progenitors shown as a 3D reconstruction, the *PLP* gene does not co-localize with SC35 imaged in the XY plane (C) or when rotated 90 degrees to the XZ view (D). The blue line outlines the nuclear periphery. Scale Bar = 5 μm.

Figure 5: Quantitation of *PLP* and *IRBP* gene nuclear localization. The *PLP* and *IRBP* genes were classified as within the peripheral region of the nucleus if the measured distance between the center of the in situ hybridization signal and the edge of the nucleus was less than 1.5 μm. When the center of the in situ hybridization signal was located within the remaining nuclear volume, the localization was counted as central. In panel A, phase contrast microscopy was used to identify the nuclear boundary. The PLP gene was more frequently

associated with the peripheral region of the nucleus in oligodendrocyte progenitors (OPs, n=22) and oligodendrocytes (oligos, n= 37). Analysis of individual IRBP alleles within each cell did not indicate a preferential association of IRBP alleles with the peripheral region of the nucleus (OPs, n=38; oligos, n=30). In panel B, both IRBP alleles were analyzed within each cell and were categorized as central-central (CC), central-peripheral (CP), or peripheral-peripheral (PP). In panel C, DAPI was used to identify the nuclear periphery. The *PLP* gene was non-randomly associated with a peripheral localization in astrocytes (astros, n=52), oligodendrocyte progenitors (OPs, n=103), or oligodendrocytes (oligos, n=101) (* $p<0.05$, chi-square). The PLP gene localization was not significantly different when comparing across cell types. Error bars = standard error of the proportion.

Figure 6: *PLP* gene nuclear localization. *PLP* genomic in situ hybridization (green or red) to detect the *PLP* gene combined with the nuclear stain DAPI (blue) to show the nuclear volume in astrocytes (A), progenitors (B), and oligodendrocytes (C). Scale bar = 10 μm.

Figure 7: Nuclear localization of SC35 splicing factors and Myt1 DNA-binding proteins in oligodendrocyte progenitors. Progenitor cultures were double immunostained using anti-SC35 detected with anti-mouse FITC (green) and anti-Myt1 detected with anti-rabbit Cy3 (red). 3D reconstruction of

SC35/Myt1 double immunostain imaged in XY plane (A) and with the 3D image rotated 90 degrees to show XZ view (B). Scale bars = 5 μm.

Figure 8: PLP genomic in situ hybridization combined with Myt1 immunostaining. Merged images for *PLP* genomic in situ hybridization (green) combined with Myt1 immunostaining (red) in pre-oligodendrocyte progenitors (A), progenitors (B), and oligodendrocytes (C). *IRBP* genomic in situ hybridization (green) merged with Myt1 immunostaining (red) in progenitors (D). Scale bars = 10 μm.

Figure 9: Quantitation of *PLP* genomic in situ hybridization combined with Myt1 immunostaining. The *PLP* gene and Myt1 domains were scored as co-localized when there was pixel overlap in the red and green channels (see Figure 8). Myt1 nuclear domains were found associated with the *PLP* gene in approximately 50% of the cells analyzed (pre-oligodendrocyte progenitors (preOP, n=50), progenitors (OPs, n=101), and oligodendrocytes (oligos, n=100). The *IRBP* gene, which is not expressed in oligodendrocyte progenitors, was also found associated with Myt1 nuclear domains in approximately 50% of the progenitor cells analyzed (n=50). No statistical difference was found between any of the groups using the chi-square statistical test. Error bars = standard error of the proportion.

Figure 10: Double genomic in situ hybridization for *MBP* and *PLP* genes. Genomic in situ hybridization with the X-linked *PLP* gene being detected with FITC-labeled *PLP* probe (green) and the autosomal *MBP* gene being simultaneously detected with a digoxigenin-labeled *MBP* probe (red). In progenitors (Figure 10A, phase contrast 10C) and in oligodendrocytes (10B, phase contrast 10D), the *PLP* and the *MBP* genes were spatially separated. Scale bar = 10 μM.

Figure 1

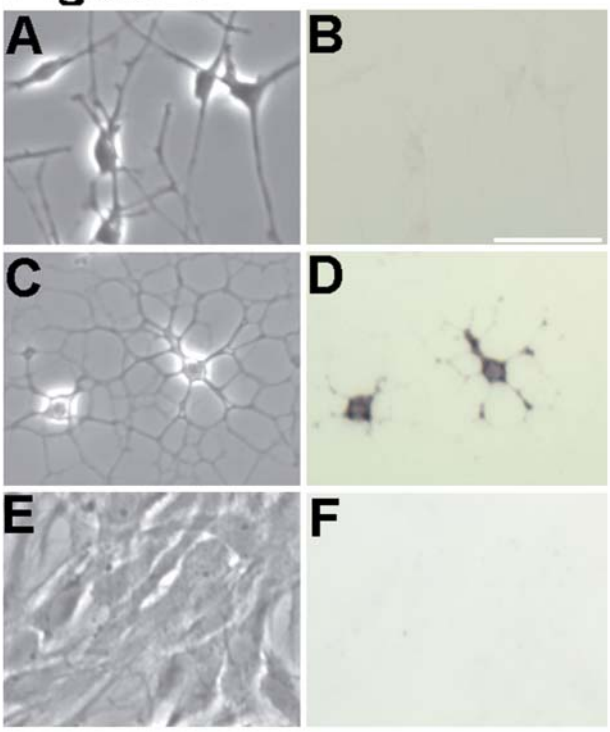

Figure 2

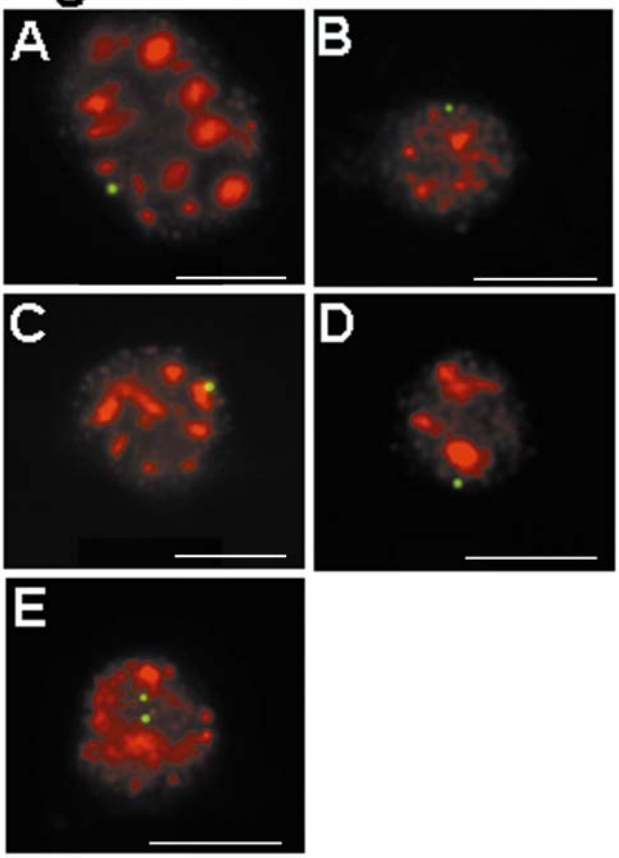

Figure 3

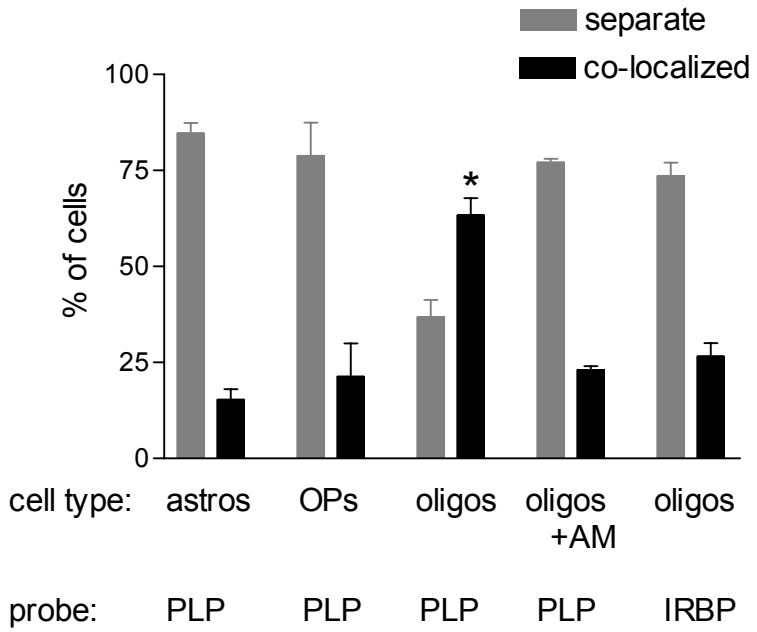
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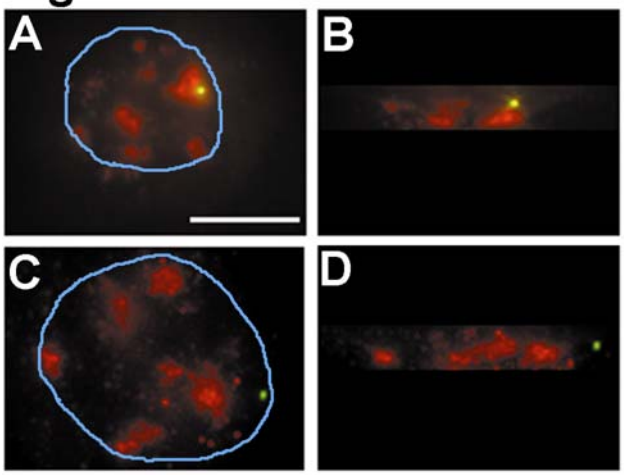
Figure 4



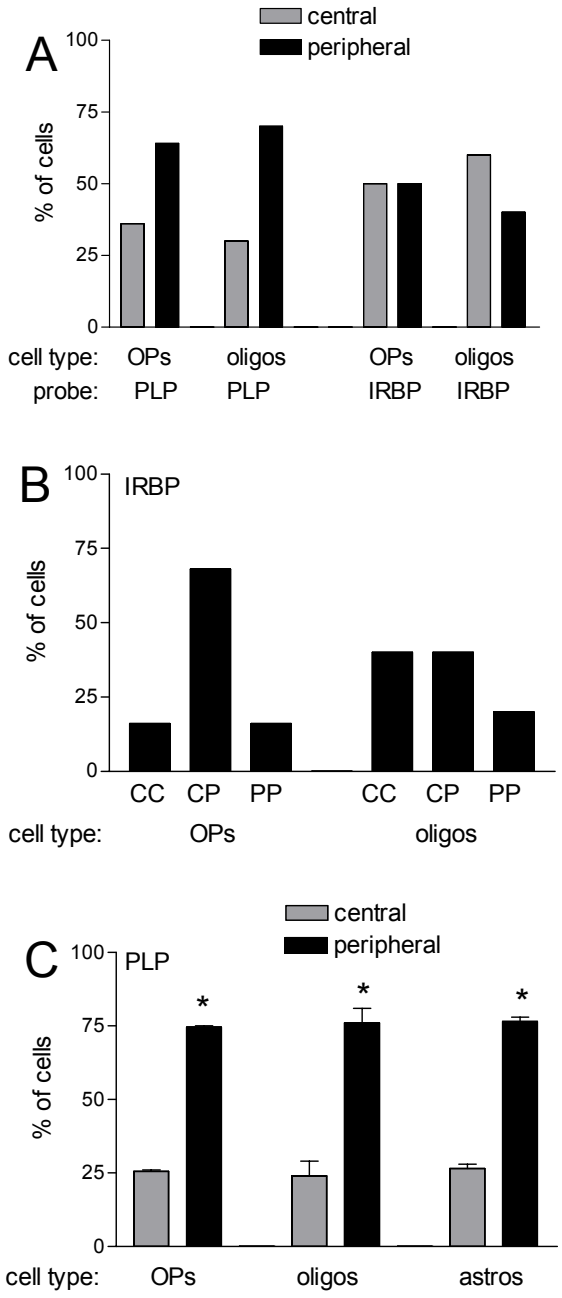

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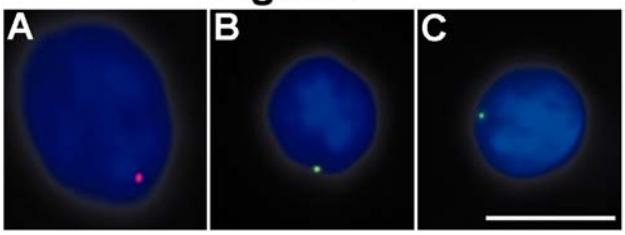
Figure 5

Figure 6



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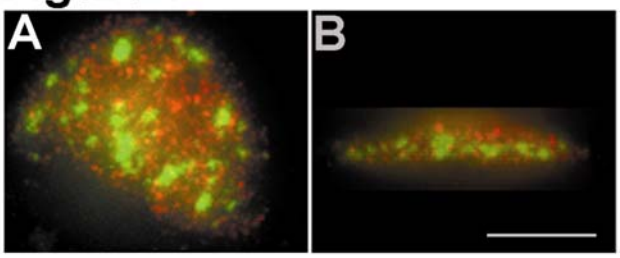

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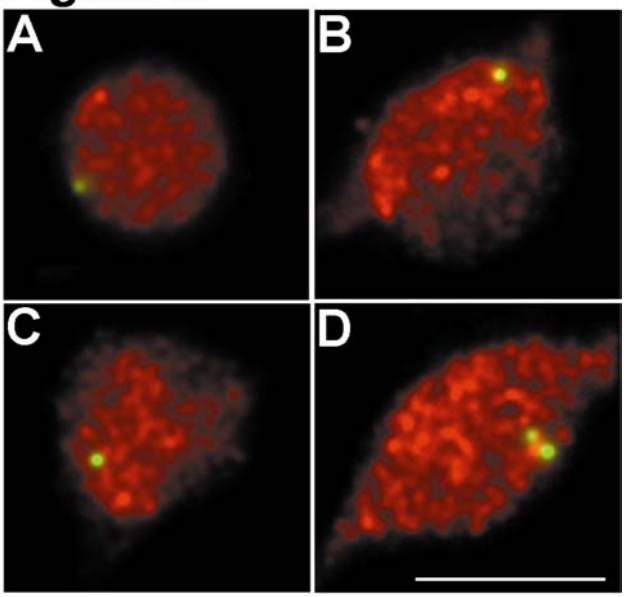

Figure 9

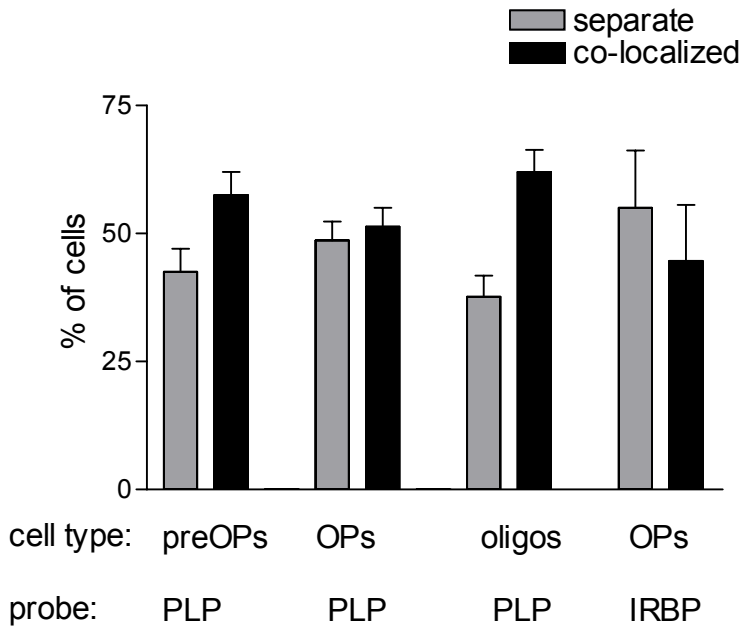
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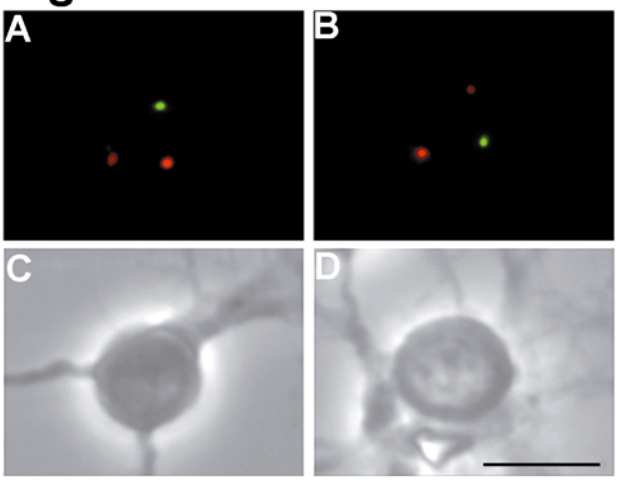
Figure 10



Paper 2

Myelin Transcription Factor 1 (Myt1) Modulates the Proliferation and Differentiation of Oligodendrocyte Lineage Cells

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Abstract

Myelin transcription factor 1 (Myt1) is a zinc-finger DNA-binding protein that is expressed in neural progenitors and oligodendrocyte lineage cells. The objective of this study was to determine the function of Myt1 by overexpressing the four zinc-finger DNA-binding domain of Myt1 (4FMyt1) that lacks the putative domains for protein-protein interaction and transcriptional activation. Expression of 4FMyt1 inhibited the differentiation of oligodendrocyte progenitors into oligodendrocytes as assessed by morphology, immunostaining, and myelin gene expression. In the presence of mitogens, oligodendrocyte progenitor proliferation was reduced by expression of 4FMyt1 as compared to the control vector. This 4FMyt1 inhibition of proliferation did not occur in astrocytes or NIH3T3 cells, which are cell types that do not express endogenous Myt1. These data indicate that Myt1 regulates a critical transition point in oligodendrocyte lineage development by modulating oligodendrocyte progenitor proliferation responses relative to terminal differentiation and myelin gene expression.

Introduction

During development, cells respond to extracellular signals which set in motion a cascade of intracellular events leading to cell fate determination and terminal differentiation (Edlund and Jessell, 1999). Neural stem cell and progenitor cell development must be appropriately regulated to generate the normal complement of neuronal, astroglial, and oligodendroglial cell types. The developmental progression that gives rise to oligodendrocytes has been well characterized in vitro and in vivo (Dubois-Dalcq and Armstrong, 1992). Aberrant oligodendrocyte development and maintenance can contribute to dysmyelinating diseases and leukodystophies (Levine et al., 2001). In these conditions, lack of normal oligodendrocytes and/or myelin sheaths results in abnormal neuronal impulse conduction and neurologic dysfunction.

Multiple extracellular signals influence oligodendrocyte lineage cell differentiation (Rogister et al., 1999). However, the intracellular molecules that mediate these effects are not well understood. As elucidated in other cell systems, extracellular signals are expected to act through intracellular cascades that in turn work through transcription factors to control expression of tissue-specific genes and terminal differentiation (Edlund and Jessell, 1999). The transcription factors Olig1 and Olig2 were recently shown to be essential for the earliest stages of the generation of oligodendrocyte lineage cells (Lu et al., 2002). However, the transcriptional control of oligodendrocyte differentiation is poorly understood. A large number of transcription factors have been identified that are expressed at various stages of oligodendrocyte development, but there

is little functional data to elucidate the role of each transcription factor in the overall differentiation program (Hudson, 2001).

One of these putative transcription factors with an expression pattern that indicates a potential role in regulating oligodendrocyte differentiation is myelin transcription factor 1 (Myt1). Myt1 is a zinc-finger DNA-binding protein that was cloned based upon affinity for a sequence in the proteolipid protein (PLP) gene promoter (Kim and Hudson, 1992). Myt1 consists of six zinc-finger domains that utilize a CCHC zinc coordination motif. The zinc-fingers are arranged with two N-terminal fingers and four C-terminal fingers, and both sets of fingers can bind independently to a site in the PLP promoter (Kim and Hudson, 1992). Myt1 contains a putative acidic transcriptional activation domain and an alpha-helical protein-protein interaction domain. In the developing central nervous system (CNS), Myt1 is expressed in the neuroepithelial germinal zones, corresponding with both neuronal and oligodendrocyte progenitors (Armstrong et al., 1995; Kim et al., 1997). Myt1 continues to be expressed in germinal zones in the adult (Armstrong et al., 1997). Following progenitor differentiation, Myt1 expression is down-regulated in terminally differentiated oligodendrocytes (Armstrong et al., 1995). Consistent with these mammalian studies, a *Xenopus* version of Myt1 was reported to promote the terminal differentiation of neurons (Bellefroid et al., 1996).

The objective of the current study was to determine the function of Myt1 in regulating oligodendrocyte lineage development. A dominant negative expression study was designed using a replication-incompetent retroviral

expression system to express the four zinc-finger DNA-binding domain of Myt1 (4FMyt1), which lacks the putative transcriptional activation domain and protein-protein interaction domain. Expression of 4FMyt1 inhibited oligodendrocyte progenitor differentiation into mature oligodendrocytes based upon analysis of cell morphology, cell type-specific antigens, and PLP mRNA transcripts. In addition, transient transfection assays showed that overexpression of either 4FMyt1 or full-length Myt1 in oligodendrocyte progenitors inhibited differentiation along the oligodendrocyte pathway. Finally, expression of 4FMyt1 inhibited proliferation of oligodendrocyte progenitors in response to mitogens. The specificity of the 4FMyt1 effect on proliferation was tested in astrocytes and NIH3T3 cells, which do not express Myt1. In the absence of Myt1 expression, 4FMyt1 had no effect on proliferation. These experiments reveal a functional impairment of critical developmental responses of oligodendrocyte lineage cells as a result of interference with Myt1 interactions.

Results

4FMyt1 localizes to the nucleus of oligodendrocyte progenitors.

Expression of a transcription factor DNA binding domain, in the absence of other functional domains, can compete with the endogenous protein for response elements and inhibit function in a dominant negative manner (O'Neill, 1995; Chen et al., 1998; Hildesheim et al., 2001). We used this rationale in designing the 4FMyt1 expression vector. The four-finger region of Myt1 has been shown to bind to the PLP promoter independently, and lacks the putative acidic transcriptional activation domain and protein-protein interaction domain (Kim and Hudson, 1992). The LNCX retroviral expression system was selected to overexpress the C-terminal four zinc-finger domain of Myt1 fused to a C-terminal FLAG epitope tag (Figure 1A). This vector employs a CMV promoter to drive a relatively high level of constitutive expression. We hypothesized that overexpressed 4FMyt1 would compete with endogenous Myt1 for the binding to response elements of target genes that regulate oligodendrocyte lineage cell functions.

Oligodendrocyte progenitors were isolated from postnatal day 2 rat pups as described in the methods, and parallel cultures were infected with 4FMyt1 and control LEGFP viral supernatants. 4FMyt1 localized to the nucleus of oligodendrocyte progenitors as determined by indirect immunofluorescence with an anti-FLAG antibody (Figure 1B, C). This nuclear localization was distinct from the control LEGFP retroviral vector, which uses the same CMV promoter to express enhanced green fluorescent protein (EGFP). EGFP fluorescence was

distributed throughout the entire cell body and processes of infected cells (Figure 1D, E).

4FMyt1 inhibits oligodendrocyte differentiation.

An effect of 4FMyt1 on oligodendrocyte differentiation was predicted because Myt1 is expressed in immature oligodendrocytes lineage cells and down-regulated after terminal differentiation (Armstrong et al., 1995; Kim et al., 1997). Oligodendrocyte progenitors were infected with retrovirus in the presence of platelet derived growth factor-AA (PDGF) and fibroblast growth factor 2 (FGF) to facilitate retrovirus infection and integration into chromatin, which requires actively dividing cells. At 48 hours post-infection, the cultures were washed to remove the PDGF and FGF, and then allowed to grow an additional 48 hours in differentiation medium, which contains T3 and insulin to promote differentiation along the oligodendrocyte pathway. At 96 hours post-infection, the cultures were fixed and infected cells were identified by FLAG immunostaining (4FMyt1 infections) or EGFP expression (control infections).

The morphology of the infected cells was determined using phase contrast microscopy. After 48 hours in differentiation medium, a majority of control LEGFP infected cells had acquired multiple branched processes typical of differentiated oligodendrocytes (Figure 1D, E). Cells infected with 4FMyt1 appeared to have fewer processes (Figure 1B, C). Fractal analysis has demonstrated that the morphological complexity of developing oligodendrocytes correlates with the stage of differentiation (Behar, 2001). Therefore, two

categories of cell morphology, oligodendrocyte and progenitor, were defined for quantitation based upon the number and complexity of cell processes (detailed in methods).

The normal regulation of oligodendrocyte progenitor differentiation by growth factors was observed in control LEGFP infections, but with a striking difference in 4FMyt1 clones. When differentiation was prevented by continuous exposure to PDGF and FGF, the cells maintained the progenitor morphology regardless of whether infected with the control LEGFP virus (15 ± 7%) or 4FMyt1 virus (10 ± 6%)(Figure 1F). After 2 days in differentiation medium, 85 ± 4% of the control LEGFP infected cells went on to develop a differentiated oligodendrocyte morphology (Figure 1F). In contrast, in parallel cultures only 39 ± 4% of 4FMyt1 infected cells developed the oligodendrocyte morphology (Figure 1F). These data indicate that 4FMyt1 expression inhibited normal progression of oligodendrocyte progenitor differentiation.

The progressive stages of oligodendrocyte lineage differentiation can be characterized more definitively by the expression of stage-specific antigens (Armstrong, 1998). The O1 monoclonal antibody recognizes a cell surface galactocerebroside, which can be used to identify terminally differentiated, post-mitotic oligodendrocytes (Sommer and Schachner, 1981; Bansal and Pfeiffer, 1992). O1 immunostaining was therefore used to determine the effect of 4FMyt1 expression on oligodendrocyte progenitor differentiation. In control LEGFP infected cultures, 33 ± 11% of the infected cells acquired O1 immunoreactivity after 48 hours in differentiation medium (Figure 2A, B, E). A dramatic difference

was observed for cells infected with 4FMyt1. Only 3 ± 1% of cells expressing 4FMyt1 had acquired O1 immunoreactivity after 48 hours (Figure 2C, D, E). Combined with the morphological analysis, these data demonstrate that very few cells expressing 4FMyt1 progressed to the mature oligodendrocyte stage of differentiation.

Expression of myelin-specific genes is another indicator of oligodendrocyte differentiation. PLP is a myelin structural protein for which mRNA levels increase as progenitors mature to differentiated oligodendrocytes. Moreover, the PLP gene is a direct target for Myt1 binding. To determine whether 4FMyt1 impaired expression of PLP mRNA, cells were infected with virus and maintained with mitogens for 24 hours. The cultures were then washed, refed with differentiation medium, and grown for an additional 72 hours to allow differentiation and expression of myelin genes. The cultures were then fixed and processed for PLP mRNA in situ hybridization and subsequent FLAG or EGFP immunostaining. The average PLP mRNA signal intensity ratio of cells infected with 4FMyt1 to non-infected cells was reduced 28 ± 9% relative to the control LEGFP ratio (Figure 3). Taken together, this analysis of multiple criteria for differentiation indicates that 4FMyt1 inhibits the differentiation of oligodendrocyte progenitors into mature oligodendrocytes.

4FMyt1 expression does not promote astrocyte differentiation.

Oligodendrocyte progenitors can differentiate along either of two pathways depending on the culture conditions. When grown in medium with T3 and insulin

such as the medium used above, the progenitors differentiate into oligodendrocytes. However, when grown in DMEM with 10% FBS, progenitors can differentiate into type-2 astrocytes (Raff et al., 1983). Since expression of 4FMyt1 inhibited the differentiation of progenitors along the oligodendrocyte pathway, we tested whether differentiation was also inhibited along the astrocyte pathway. Cultures of progenitors were infected with 4FMyt1 or control LEGFP virus and maintained in PDGF and FGF for 48 hours before being switched for an additional 48 hours to medium that promoted astrocyte differentiation (DMEM with 10% FBS) or oligodendrocyte differentiation (as above). The cells were then fixed and immunostained for glial fibrillary acidic protein (GFAP), an intermediate filament protein that distinguishes astrocytes (Raff et al., 1983). When progenitor cultures were grown in astrocyte differentiation medium (Figure 4A), a similar majority of control LEGFP or 4FMyt1 expressing cells were immunolabeled with GFAP (Figure 4A, C). Therefore, 4FMyt1 did not inhibit astrocytic differentiation of progenitors. When grown in oligodendrocyte differentiation medium, 24 $\pm$ 6% of the cells infected with control LEGFP virus were GFAP positive while 35 $\pm$ 5% of the cells infected with 4FMyt1 virus were GFAP positive (Figure 4B, C). These data indicate that 4FMyt1 inhibition of oligodendrocyte maturation is not due to a significant shift toward an astrocyte cell fate, but rather maintenance of a progenitor phenotype as expected from our morphological analysis. These experiments also demonstrate that 4FMyt1 specifically inhibits differentiation along the oligodendrocyte pathway.

Expression of 4FMyt1 inhibits proliferation of oligodendrocyte progenitors. Endogenous Myt1 is expressed by immature oligodendrocyte lineage cells that are highly proliferative. Since retroviral infections lead to stable viral integration and reporter gene expression, the number of cells per clone serves as a measure of proliferation of the daughter cells subsequent to infection. To determine whether 4FMyt1 interfered with proliferation, oligodendrocyte progenitors were infected with the control LEGFP or 4FMyt1 virus and then allowed to grow in the presence of PDGF and FGF mitogens for 48 hours. Cultures were refed either fresh medium with PDGF and FGF, or were washed and refed differentiation medium. At 96 hours post-infection the cultures were fixed and the clone size was determined by counting the number of cells per clone (defined in the methods). In cultures of cells infected with the control LEGFP virus and maintained in mitogens throughout the 96-hour growth period, the mean clone size was 5.5 cells/clone (Figure 5A, D). In parallel cultures infected with 4FMyt1, the mean clone size was significantly reduced to 4.2 cells/clone (Figure 5D).

Both proliferation and differentiation effects on progenitors can contribute to changes in clone size. If infected progenitors are induced to differentiate into post-mitotic oligodendrocytes, they will not undergo further rounds of division so that the number of cells per clone will be reduced. This effect of differentiation is clearly demonstrated by the fact that in cultures switched to differentiation medium for 48 hours, there were fewer cells/clone with no difference in clone size between control LEGFP and 4FMyt1 infected cultures (Figure 5D).

However, since cells expressing 4FMyt1 maintained a progenitor phenotype (Figure 1F), 4FMyt1 inhibition of proliferation in the presence of mitogens was independent of an effect on differentiation.

4FMyt1 does not inhibit proliferation of cell types that do not express Myt1. To test the specificity of the 4FMyt1 effect relative to endogenous Myt1 expression, clone size was a more feasible means of analysis than differentiation, which would be more difficult to quantify in other cell types. Astrocytes and NIH3T3 cells were examined since both do not express detectable Myt1. Astrocyte cultures were infected with control and 4FMyt1 virus, and allowed to grow for 96 hours. Control LEGFP infected astrocytes were identified by EGFP expression and 4FMyt1 infected astrocytes were identified by FLAG immunostaining (Figure 6D). Myt1 immunostaining confirmed the absence of detectable Myt1 expression in both infected and non-infected astrocytes (Figure 6E, Armstrong et al., 1995). Myt1 immunostaining of oligodendrocyte cultures was performed in combination with FLAG immunostaining (Figure 6A) as a positive control to confirm appropriate detection of endogenous Myt1 (Figure 6B), and demonstrates partial co-localization of 4FMyt1 with endogenous Myt1 (Figure 6C). Control LEGFP infected astrocyte clones had a mean number of cells/clone of 2.7, while 4FMyt1 infected astrocyte clones had a mean of 2.3 cells/clone (Figure 5D). Similar experiments were performed in NIH3T3 cells, in combination with Myt1 immunostaining again confirming the absence of detectable Myt1 expression (data not shown). In NIH3T3 cultures the clones

infected with the control LEGFP virus had a mean of 8.2 cells/clone, while the 4FMyt1 infected clones had a mean of 8.7 cells/clone (Figure 5D). In both the astrocytes and NIH3T3 cells, there was no significant difference between control LEGFP infected and 4FMyt1 infected clones. These data demonstrate that in the absence of endogenous Myt1 expression 4FMyt1 does not effect proliferation, indicating that 4FMyt1 is specifically antagonizing endogenous Myt1 function.

Oligodendrocyte progenitor motility is not impaired by 4FMyt1.

Another major physiological response of oligodendrocyte progenitors is migration or motility. Progenitor migration is stimulated by PDGF and FGF (Armstrong et al., 1990; Simpson and Armstrong, 1999). Therefore dispersion of cohort cells within retrovirally-labeled clones was used to estimate progenitor motility while expressing control LEGFP or 4FMyt1. Cohort dispersion was measured as the mean distance between cells within each clone (see methods). There was no statistically significant difference in the mean distance of dispersion among cohort cells between control LEGFP and 4FMyt1 infected clones grown in PDGF and FGF for 96 hours (Figure 7). The motility in response to PDGF and FGF demonstrates that progenitor viability is maintained while 4FMyt1 is expressed. The lack of effect on cohort dispersion also emphasizes that 4FMyt1 inhibition of differentiation and proliferation responses is specifically inhibiting only a subset of progenitor functional responses.

Overexpression of full-length Myt1 inhibits differentiation.

To assess the effects of full-length Myt1 expression on oligodendrocyte progenitor differentiation, retroviral expression was attempted. Unfortunately, it was not possible to produce infectious virions expressing full-length Myt1 (detailed in methods). To solve the retroviral expression problem for full-length Myt1 and test the effects of control LEGFP and 4FMyt1 in a second assay, transient transfections of oligodendrocyte progenitors with plasmid-encoded Myt1 were performed. This analysis focused on the O1 differentiation assay, which gave the most dramatic effect with the retroviral approach. Cultures of oligodendrocyte progenitors were transfected while in medium with PDGF and FGF, and then switched to oligodendrocyte differentiation medium for 48 hours. Only 3% of 4FMyt1 and 2% of Myt1 transfected cells acquired O1 positive immunoreactivity compared to 33% of control LEGFP transfected cells (Figure 8). The data for control LEGFP and 4FMyt1 correlate well with the data obtained in the retroviral expression studies. Furthermore, overexpression of either full-length Myt1 or 4FMyt1 prevented oligodendrocyte progenitors from progressing to the mature oligodendrocyte stage of differentiation.

Discussion

The balance between cell proliferation and differentiation plays a critical role in development, regeneration, and tumor formation. Based upon our previous studies of Myt1 expression in the developing CNS and in gliomas (Armstrong et al., 1995; Armstrong et al., 1997; Kim et al., 1997), the present study was designed to test the hypothesis that Myt1 may potentially regulate oligodendrocyte lineage proliferation and differentiation. Oligodendrocyte primary cell cultures were used to assess the function of Myt1 in oligodendrocyte lineage cell differentiation, proliferation, and migration. Retroviral expression of the four zinc-finger DNA-binding domain of Myt1, i.e. 4FMyt1, had multiple effects on oligodendrocyte function. In medium that promoted differentiation, expression of 4FMyt1 interfered with the differentiation of oligodendrocyte progenitors as assessed by three criteria: morphology, O1 immunoreactivity, and PLP mRNA expression. In addition to the effect of 4FMyt1 on differentiation, expression of 4FMyt1 reduced the proliferation of oligodendrocyte progenitors in response to potent mitogens. The inhibitory effects of 4FMyt1 were specific to differentiation and proliferation in that oligodendrocyte progenitor migration did not appear to be impaired.

Several lines of evidence prompted us to examine the effect of Myt1 on oligodendrocyte differentiation. Myt1 is expressed in embryonic, early postnatal, and adult germinal zones in both neuronal and oligodendrocyte progenitors (Armstrong et al., 1995; Kim et al., 1997). In oligodendrocyte lineage cells, Myt1 expression is down-regulated after PLP accumulates in differentiated

oligodendrocytes (Armstrong et al., 1995). The temporal and spatial expression pattern of Myt1 in the oligodendrocyte lineage indicates that Myt1 might play a role in regulating terminal differentiation. Furthermore, both the N-terminal set of two zinc-fingers and the C-terminal set of four zinc-fingers of Myt1 can bind independently and specifically to the promoter of the PLP gene, which is a myelin structural gene that is transcriptionally up-regulated upon differentiation of oligodendrocytes. Consistent with these findings in the oligodendrocyte lineage, a study of *Xenopus* Myt1 showed inhibition of neuronal differentiation with overexpression of several regions of Myt1, including the central domain, the two zinc-finger domain, and the four zinc-finger domain (Bellefroid et al., 1996). In the present study, inhibition of oligodendrocyte differentiation was also observed with overexpression of either the four-finger DNA-binding domain or full-length Myt1.

Analysis of full-length Myt1 overexpression required the use of transient transfections in primary oligodendrocyte lineage cultures, since retroviral infections with full-length Myt1 could not be demonstrated using the retroviral system. In these transient transfection experiments, the control LEGFP and 4FMyt1 plasmids yielded very similar results as was shown with the retroviral expression approach. Surprisingly, the anti-FLAG localization of transfected full-length Myt1 demonstrated predominantly cytoplasmic immunoreactivity with a lower level of nuclear signal in most of the transfected cells. This result indicates that either the nuclear import or export of Myt1 may be a regulated process, and that the subcellular distribution of Myt1 may be sensitive to overexpression. The

Myt1 sequence has putative nuclear localization sequences in the 4FMyt1 region and a potential nuclear export signal sequence in the C-terminal region not contained within the 4FMyt1 sequence (Boulikas, 1994; Hamilton et al., 2001). Furthermore, a nuclear to cytoplasmic transition of Myt1 immunoreactivity has been shown to be developmentally regulated (Armstrong et al., 1995).

One possible explanation for the similar effects of full-length Myt1 and 4FMyt1 maybe a limiting availability of other factors that are required for the normal function of Myt1. In this scenario, excess 4FMyt1 or full-length Myt1 could act as a dominant negative by disrupting the balance of binding interactions or the formation of complexes involving these factors, Myt1, and promoter/enhancer sites. This mechanism has been proposed to occur with the transcriptional corepressor NCoR and the retinoic acid receptor. Transfection of NCoR deletion mutants lacking an interaction site for mSin3 caused a loss of repression, as expected for the dominant negative. However, transfection of full-length NCoR also caused a loss of repression indicating that overexpressed NCoR may be titrating out a cofactor required for repression (Soderstrom et al., 1997).

Myt1 expression correlates with the proliferative cell types of the oligodendrocyte lineage in the developing and adult CNS. In addition Myt1 is up-regulated in gliomas, in which increased Myt1 expression was positively correlated with the Ki-67 proliferation marker (Armstrong et al., 1997). The present results with 4FMyt1 provide further evidence that Myt1 regulates oligodendrocyte lineage cell proliferation. The specificity of this 4FMyt1 effect is

demonstrated by the lack of effect on the proliferation of astrocytes and NIH3T3 cells, which do not express endogenous Myt1. Myt1 may regulate the expression of, or directly interact with, a cell cycle progression protein. Since the consensus DNA-binding site for Myt1 predicts a relatively broad range of potential target sequences (Hudson, 2001), it is not surprising that Myt1 might regulate multiple target genes and influence multiple cellular functions. However, this consensus does not help yet in predicting targets of Myt1 involvement in cell proliferation.

Various signaling mechanisms initiate intracellular signaling cascades that ultimately lead to changes in gene expression and regulate oligodendrocyte functions (Rogister et al., 1999). The multiple functions of Myt1 on proliferation and differentiation could be explained by differences in the extracellular signals and/or the availability of molecules that interact with Myt1. There is evidence that growth factors can control the nuclear localization of proteins such as Id2, which is a transcriptional repressor involved with regulating differentiation in multiple cell types including oligodendrocyte lineage cells (Wang et al., 2001). Additionally, the complement of transcription factors that is present in oligodendrocyte progenitors is clearly different than in mature oligodendrocytes. For example, thyroid hormone receptor β1 is highly expressed in differentiated oligodendrocytes relative to oligodendrocyte progenitors (Baas et al., 1994). There are numerous other examples of transcription factors that are more highly expressed in progenitors relative to oligodendrocytes (Hudson, 2001). Gene regulation is thought to occur through the combinatorial effect of multiple

regulatory proteins assembling on the promoter and enhancers of a target gene. Therefore, a combination of extracellular signals and the complement of transcription factors present at a given stage of differentiation might account for the ability of 4FMyt1 to influence both proliferation and differentiation.

Myt1 expression in vivo and the current functional data indicate that Myt1 regulates a critical transition in oligodendrocyte lineage development in modulating the oligodendrocyte progenitor proliferative response relative to terminal differentiation and myelin gene expression. Identification of partners interacting with Myt1 and target genes regulated by Myt1 will be important for better understanding the function of Myt1 in neural cell development. This work would then provide further insight into the potential role of Myt1 in pathological conditions in which Myt1 is up-regulated in the adult CNS, such as in some cases of multiple sclerosis (unpublished observation), gliomas (Armstrong et al., 1997), and spinal cord injury (Wrathall et al., 1998).

Materials and Methods

Cell culture

Primary cultures from neonatal rat brains were prepared as previously described (Armstrong et al., 1995; Armstrong, 1998). Briefly, postnatal day 2 rat brains were dissociated, plated in tissue culture flasks, and allowed to grow for 7-10 days. The flasks were placed on a rotary shaker to dislodge immature oligodendrocyte lineage cells, which were then plated onto poly-D-lysine coated chamber slides for PLP mRNA detection and 24-well plates for proliferation, differentiation, and dispersion analysis. Oligodendrocyte progenitors cells were grown in Silberberg's defined medium (Eccleston and Silberberg, 1984) which contains high insulin (5 μg/ml) and T3 (Triiodothyronine; 30 nM) and supplemented with 0.5% FBS. Under these conditions, the majority of the progenitors differentiate into mature oligodendrocytes over 48 hours in culture. The majority of these cells maintain the progenitor phenotype when grown in defined medium with PDGF (10 ng/ml) and FGF (10 ng/ml)(both from R and D Systems, Minneapolis, MN). Astrocytes were obtained from the same primary rat brain glial cultures by purification of the population that remained adhered to the initial flasks after the oligodendrocyte lineage cells were dislodged. Astrocytes were passaged and grown in DMEM with 10% FBS. NIH3T3 cells (ATCC; Manasses, VA) were also grown in DMEM with 10% FBS. All animals were handled in accordance with procedures approved by the USUHS Institutional Animal Care and Use Committee.

Retroviral Vector Construction and Infections

High fidelity PCR using PWO polymerase (Roche Applied Science; Indianapolis, IN) was used to amplify the 4FMyt1FLAG sequence (containing FLAG and amino acids 730-942 of Myt1 from plasmid RA8, unpublished) and create NdeI and XhoI cut sites for ligation into pDNR-3 (Clontech; Palo Atlo, CA). pDNR-3 was then sequenced to confirm the reading frame and sequence of the 4FMyt1FLAG insert. The donor vector was combined with the pLP-LNCX retroviral expression vector, in the presence of *cre* recombinase to transfer the 4FMyt1FLAG cassette into the retroviral vector according to the manufacturer's instructions (Clontech; Palo Atlo, CA). Similarly, full-length Myt1 (LDN145 clone L28; unpublished, L.D.H.) was cut with EcoRI and ligated into pDNR3 and then transferred to pLP-LNCX. The retroviral plasmids were then transfected into Eco2-293 packaging cells (Clontech; Palo Atlo, CA) with Lipofectamine 2000 (Invitrogen; Carlsbad, CA). Transfections were performed with Booster 1 (Genetherapy; San Diego, CA) and 4 μg of DNA per 35mm dish. At 6 hours post-transfection, the medium was removed and replaced with DMEM with 10% FBS. At 48 hours post-transfection the supernatant containing the packaged virions was filtered through a 0.45-micron cellulose acetate filter and used as viral stocks for infections.

Initial infections were performed in NIH3T3 cells to determine the titer of the retroviral stock supernatants. A dilution series was performed and the number of clones counted in the furthest dilution was used to determine clone forming units/ml (CFU/ml). The CFU/ml of the retroviral stocks was used to calculate the dilution required to achieve less than 10 infections per well of a 24-

well plate. The control pLEGFP plasmid typically yielded higher titer supernatants ($1.0X10^6$ CFU/ml), which were then diluted with supernatant from a mock-transfected dish to achieve the same CFU/ml in the control LEGFP and 4FMyt1 viral stocks. Polybrene was added to the supernatant to a final concentration of 6 μg/ml. For infection of progenitor cells, PDGF and FGF were added to a 10 ng/ml final concentration in the infection medium.

Additional techniques were attempted to try to produce infectious virions expressing full-length Myt1. The correct coding sequence of Myt1 was confirmed within the retroviral vector. Myt1 expression in transfected packaging cells was confirmed using the FLAG antibody and an antibody that recognizes the central domain of Myt1 (data not shown). Production of infectious virions expressing full-length Myt1 was attempted in several different packaging cell lines without success, even though infections were produced for another gene insert of similar size in the same vector. Additional packaging cell lines and transfection reagents were tested including the packaging cell lines Eco-293 and GP293 (Clontech; Palo Atlo, CA) and Phoenix eco packaging cells (Orbigen; San Diego, CA). Transfection reagents tested included Lipofectamine, Lipofectamine 2000 and Fugene (Roche Applied Science, Indianapolis, IN). These results led us to suspect there may be a sequence-specific problem with full-length Myt1 that interfered with this retroviral approach.

Primary Oligodendrocyte Transfections

Oligodendrocyte progenitors were plated as described above at a seeding density of 60,000 cells per well of a 24-well dish. The following day, the cultures were transfected in triplicate with 33 μl/well of transfection mixture [6 μl of Fugene (Roche Applied Science; Indianapolis, IN) per 1 μg of DNA in 100 μl of DMEM]. At 24 hours post-transfection, the cultures were washed and refed differentiation medium for an additional 48 hours prior to fixation.

Proliferation, Differentiation, and Dispersion Assays

Oligodendrocyte progenitors were plated in defined medium (see above) with a final concentration of 0.5% FBS and 10 ng/ml PDGF and 10 ng/ml FGF. After growing overnight, the medium was replaced with retroviral stock supernatant. After incubation with retrovirus for 6 hours, the cultures were washed once, and transferred to defined medium/0.5% FBS with PDGF and FGF. At 48 hours post-infection the medium was changed to fresh defined medium/0.5% FBS with or without PDGF and FGF, as noted in results. At 96 hours post-infection the cells were fixed with 4% paraformaldehyde.

Clonal analysis was used to examine oligodendrocyte progenitor differentiation, proliferation, and dispersion. A low multiplicity of infection was used such that there were approximately 10 clones per well of a 24-well plate in which 20,000 progenitors had been seeded. A clone was defined as a group of labeled cells surrounded by a border, of at least 600 microns in width, that had no labeled cells. The number of cells per clone was used to estimate

proliferation during the 96-hour period. In the morphological analysis using phase contrast microscopy, cells were scored as progenitors if they had less than or equal to 4 non-branched processes. Cells were scored as oligodendrocytes if the cell had greater than or equal to 4 processes with extensive branching. In the cell dispersion analysis the mean distance between cohort cells within each clone was calculated by measuring the distance from each cohort cell within a clone to the other cohort cells within the clone, and then repeating for all cells within that clone.

Immunostaining

After fixation with 4% paraformaldehyde, cells were permeabilized with 0.05% Triton-X 100 for 5 minutes. Anti-FLAG M2 (mouse IgG)(Sigma; St. Louis, MO) was added at a 1:2000 dilution and incubated overnight at 4° C. Following blocking with 10% normal donkey serum, the primary anti-FLAG antibody was detected with donkey anti-mouse IgG-Cy3 or FITC. To identify mature oligodendrocytes, some cultures were double immunolabeled with FLAG and O1 (mouse IgM). The FLAG/O1 double immunostain was carried out sequentially with O1 immunolabeling following the FLAG detection. O1 was detected with donkey anti-mouse IgM-FITC or Cy3. To identify astrocytes, GFAP was immunolabeled with rabbit anti-GFAP at a 1:100 dilution (rabbit IgG)(DAKO; Carpenteria, CA) and detected with donkey anti-rabbit FITC or Cy3. Rabbit α-Myt1-His polyclonal antibody raised against the central domain (amino acids 541-727) was used to immunolabel endogenous Myt1 (Armstrong et al., 1995). All

secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA).

PLP mRNA in situ hybridization

In situ hybridization for PLP mRNA was performed as previously described (Redwine and Armstrong, 1998). Briefly, cells were fixed with 4% paraformaldehyde, acetylated, and prehybridized with RNA hybridization buffer (DAKO; Carpenteria, CA). A 980 bp cDNA corresponding to the entire coding region of the mouse PLP gene, derived from pLH116 (Hudson et al., 1987), served as a template to incorporate digoxigenin-11-UTP (Roche Applied Science, Indianapolis, IN) using in vitro transcription (Ambion; Austin, Texas). The riboprobe was denatured and allowed to hybridize overnight. The riboprobe hybridization was detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Applied Science, Indianapolis, IN) followed by a 2-hour NBT/BCIP colorimetric substrate reaction (DAKO; Carpenteria, CA). The NBT/BCIP colorimetric reaction can proceed up to several days (Breitschopf et al., 1992). In our system, we experimentally determined the 2-hour time point to be prior to saturation for the PLP in situ hybridization NBT-BCIP colorimetric reaction (data not shown). The colorimetric reaction was stopped by washing in water, and the cells were then processed for immunolabeling with anti-FLAG (see above) or anti-GFP at 1:100 dilution (mouse IgG)(Clontech; Palo Atlo, CA). Semi-quantitative analysis of relative PLP mRNA expression was performed with Metamorph software. The average signal intensity for infected cells and multiple

adjacent non-infected cells in both control LEGFP and 4FMyt1 infected cultures was measured. The infected to non-infected signal intensity ratio was calculated for each field.

Image Collection and Statistical Analysis

Images were collected with an Olympus IX70 epifluorescence microscope equipped with a 20X objective using a Spot2 digital camera. The images were analyzed using Metamorph software (Universal Imaging Corporation; West Chester, PA), and figures were prepared in Adobe Photoshop (Adobe; San Jose, California). All quantitation was based on data combined from at least 3 independent preparations of cells from separate litters of animals. Chi-square statistical analysis was used to compare means from the differentiation experiments, and the independent Students t-test was used to compare means in the proliferation, dispersion, and PLP mRNA expression experiments.

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Abbreviations: CNS-central nervous system, CFU-clone forming units, DMEM-Dulbecco's Modified Eagle Medium, FBS-fetal bovine serum, FGF-fibroblast growth factor 2, Myt1-myelin transcription factor 1, PDGF-platelet derived growth factor-AA, PLP-proteolipid protein.

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Figure Legends

Figure 1: Retroviral vector design and expression. Expression vectors (A) created in the pLP-LNCX retroviral vector contain 5' and 3' long terminal repeats (LTR) regions for virus integration, a retroviral packaging signal (PS), and the cytomegalovirus promoter (pCMV) to drive constitutive expression. The control LEGFP virus expresses enhanced green fluorescent protein (EGFP). The experimental vectors express the four-finger region of Myt1 (4FMyt1) or full-length Myt1 as a fusion with the FLAG epitope tag (FG). Exogenous expression of 4FMyt1 localizes to the nucleus of oligodendrocyte progenitors (B; red). The phase contrast image of the same field as B, shows bipolar processes that are characteristic of progenitors (C). Control LEGFP infected oligodendrocytes are identified by EGFP expression throughout the cell body and processes (D; green). Phase contrast image of the same field as D shows multiple branched processes that are characteristic of differentiated oligodendrocytes (E). Quantitation of morphology of infected cells is shown in F. Cells were scored as progenitors or as oligodendrocytes based on the number of processes and complexity (see methods). In medium with platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF), the cells remain mainly progenitors (N = 6 independent experiments; 285 cells expressing LEGFP, 231 cells expressing 4FMyt1). Expression of 4FMyt1 reduced the percentage of cells that differentiated into oligodendrocytes in defined medium with T3 and insulin but without PDGF and FGF (N = 6 independent experiments, 143 cells expressing

LEGFP, 248 cells expressing 4FMyt1)(error bars = standard error of the proportion; * p<0.0001, chi-square). Scale bar = 10 microns.

Figure 2: O1 immunolabeling of cells infected with 4FMyt1 and control LEGFP. A control LEGFP infected oligodendrocyte (A; green) showing O1 immunostaining of processes and membrane sheets (B; red). A 4FMyt1 infected oligodendrocyte progenitor immunolabeled with FLAG (C; green) is negative for O1 immunostaining (D; red). Quantitation of cells scored for O1 immunoreactivity is shown in panel E. In progenitors that were infected with 4FMyt1, there is a significant reduction in the percentage of cells that become oligodendrocytes and acquire O1 immunoreactivity (N = 3 independent experiments, 72 cells expressing LEGFP, 133 cells expressing 4FMyt1)(error bars = standard error of the proportion; * p<0.0001, chi-square). Scale bar = 10 microns.

Figure 3: PLP mRNA in situ hybridization combined with FLAG immunostaining. Control LEGFP infected oligodendrocyte showing PLP mRNA in situ hybridization as blue/black NBT/BCIP colorimetric reaction (A; brightfield), and EGFP immunostaining in red (B; fluorescence of same field as A). 4FMyt1 infected cell showing PLP mRNA in situ hybridization (C; brightfield), and FLAG immunostaining in red (D, fluorescence of same field as C). An infected cell in panel C is identified by the large arrow and a non-infected cell is identified by a small arrow. Quantitation of PLP mRNA expression assay is shown in E. Brightfield images were converted to grayscale and the ratio of

infected to non-infected average signal intensity was calculated for each field containing infected cells. There is a significant decrease in the average signal intensity ratio in cells infected with 4FMyt1 compared to control LEGFP infections (N = 4 independent experiments, 50 cells expressing LEGFP, 101 cells expressing 4FMyt1)(error bars = standard error of the mean; * $p<0.05$, t-test). Scale bar = 10 microns.

Figure 4: GFAP immunolabeling of cells infected with 4FMyt1 and control LEGFP. Progenitor cultures were grown in either DMEM with 10% FBS to promote differentiation along the astrocytic pathway (A) or defined medium with T3 and insulin to promote differentiation along the oligodendrocyte pathway (B). Merged fluorescent image of infected astrocytes expressing LEGFP (A; green) and GFAP immunoreactivity (A; red). Merged fluorescent image of infected oligodendrocyte expressing 4FMyt1 detected with FLAG immunostaining (B; red) and a non-infected astrocyte immunolabeled with GFAP (B; green). Quantitation of infected cells scored for GFAP immunoreactivity is shown in C. Expression of LEGFP (LE) or 4FMyt1 (4F) does not alter GFAP expression in medium that promotes differentiation of astrocytes or oligodendrocytes (Oligo). (N = 3 independent experiments. Astrocyte 10% FBS: 85 cells expressing LEGFP, 108 cells expressing 4FMyt1. Oligo differentiation medium: 131 cells expressing LEGFP, 165 cells expressing 4FMyt1)(error bars = standard error of the proportion). Scale bar = 10 microns.

Figure 5: Proliferation analysis of cells expressing control LEGFP and 4FMyt1. Examples of a control LEGFP infected clone of oligodendrocyte progenitors (A; green). A 4FMyt1 infected clone of oligodendrocytes (B; red), with phase contrast image (C) of the same field as B. Quantitation of the number of cells/clone is shown in D. In medium containing PDGF and FGF (+PF) to maintain proliferation of progenitors (prog), there is a significant decrease in the number of progenitor cells/clone with 4FMyt1 (4F) infections compared to the control LEGFP (LE) infections (* $p<0.05$, t-test)(N = 6 independent experiments, 465 cells expressing LEGFP, 337 cells expressing 4FMyt1). In the absence of these mitogens (-PF), the progenitors differentiate into postmitotic oligodendrocytes (oligo)(N = 6 independent experiments, 60 cells expressing LEGFP, 95 cells expressing 4FMyt1). For the infections of astrocytes (astro)(N = 4 independent experiments, 104 cells expressing LEGFP, 75 cells expressing 4FMyt1) or NIH3T3 cells (N = 3 independent experiments; 229 cells expressing LEGFP, 331 cells expressing 4FMyt1), there is no significant difference in the number of cells/clone between expression of control LEGFP and 4FMyt1)(error bars = standard error of the mean). Scale bar = 20 microns.

Figure 6: Immunostaining of Myt1 in cells expressing 4FMyt1. Oligodendrocyte progenitor double immunolabeled to detect 4FMyt1 with anti-FLAG (A; green) and endogenous Myt1 immunostaining with α-Myt1His antibody (B, red). The merge of images A and B shows partial co-localization of overexpressed 4FMyt1 with endogenous Myt1 (C; co-localization appears

yellow). 4FMyt1 infected astrocytes detected with FLAG immunostaining (D; green), corresponding negative Myt1 immunostaining (E; red), and phase contrast image (F). Scale bar = 10 microns.

Figure 7: Dispersion of oligodendrocyte progenitors. Clone of 4FMyt1 infected oligodendrocyte progenitors in red (A) with the measurement lines used to calculate the mean distance between cohort cells (see methods). Quantitation of clone dispersion using mean distance between cohort cells within each clone is shown in B. In medium containing PDGF and FGF to induce motility, there is no significant difference in the mean distance between cohort cells within clones infected with 4FMyt1 and control LEGFP infected cells (N = 3 independent experiments, 20 clones expressing LEGFP, 22 clones expressing 4FMyt1)(error bars = standard error of the mean). Scale bar = 10 microns.

Figure 8: O1 immunolabeling of transiently transfected cells. Oligodendrocyte progenitor transfected with full-length Myt1 (A; red) with DAPI staining to identify cell nuclei (B; blue). For the same cells, negative O1 immunostaining is shown (C; green). The merged image of A and B shows the predominantly cytoplasmic localization of transfected Myt1 (D), with the phase contrast image (E). Quantitation of the transient transfection expression experiments is shown in F. There is a significant decrease in the percent of O1 immunolabeled cells that were transfected with either 4FMyt1 or full-length Myt1 compared to the control LEGFP transfected cells (N = 3 independent

experiments, 307 LEGFP transfected cells, 202 4FMyt1 transfected cells, 109 full-length Myt1 transfected cells)(error bars = standard error of the proportion; $p<0.05$, chi-square). Scale bar = 10 microns.

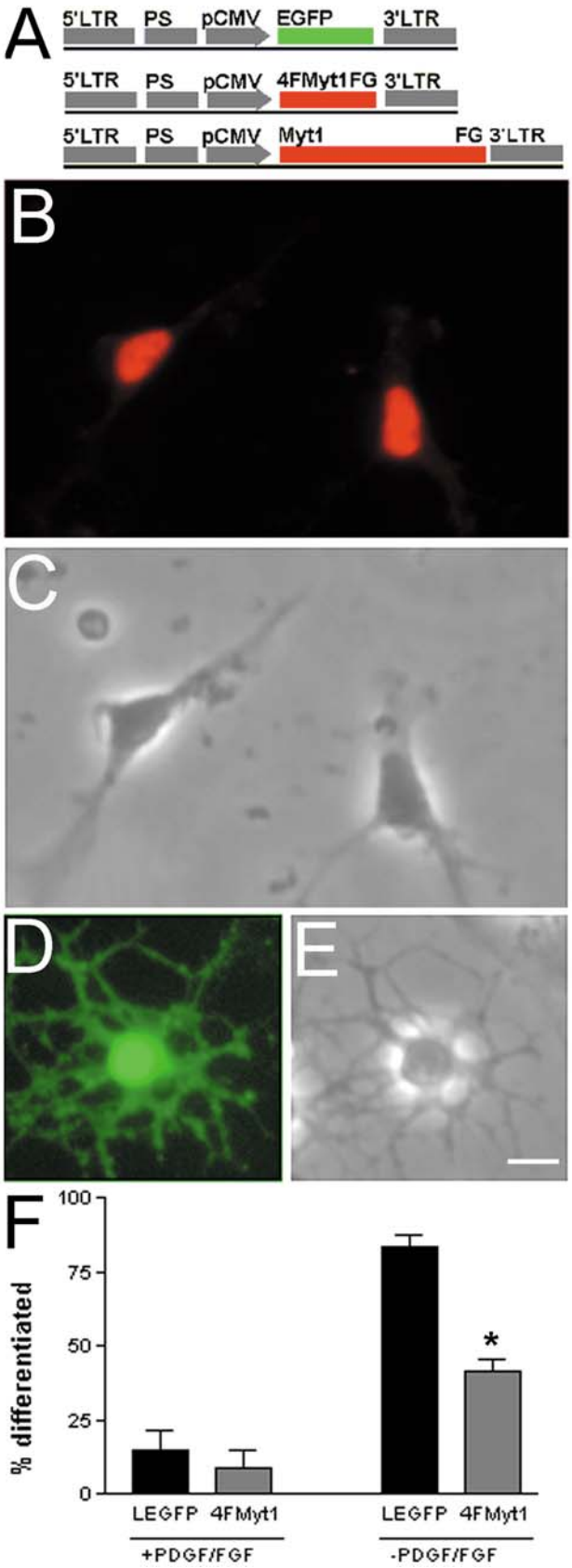

A
5'LTR
PS
pCMV
EGFP
3'LTR
5'LTR
PS
pCMV
4FMyt1FG
3'LTR
5'LTR
PS
pCMV
Myt1
FG
3'LTR
B
C
D
E
F
% differentiated
100
75
50
25
0
*
LEGFP
4FMyt1
LEGFP
4FMyt1
+PDGF/FGF
-PDGF/FGF

Figure 1

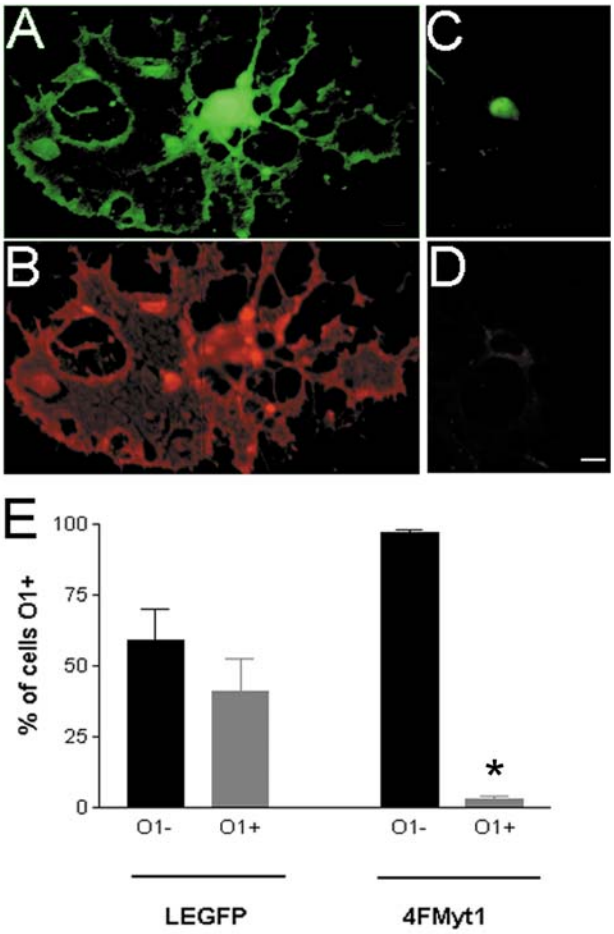


Figure 2

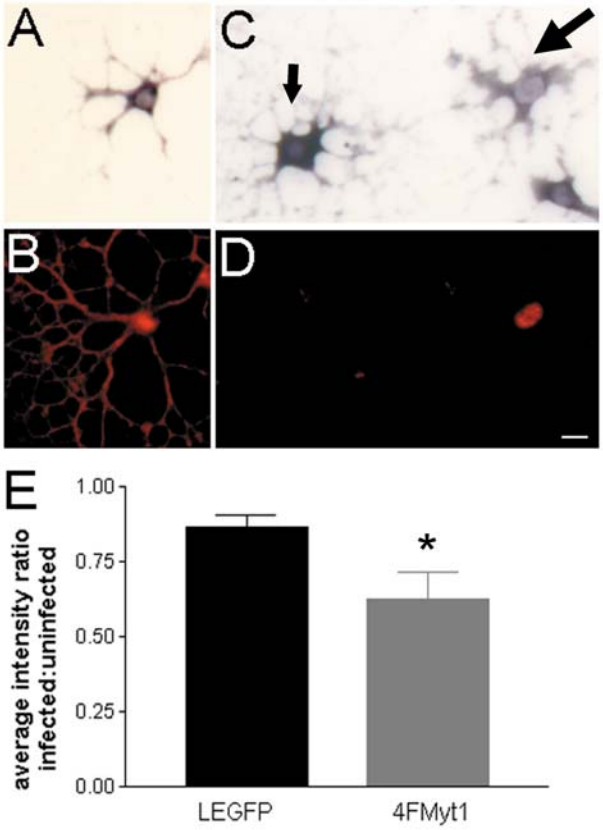

A
C
B
D
E
1.00
0.75
0.50
0.25
0.00
average intensity ratio
infected:uninfected
*
LEGFP
4FMyt1

Figure 3

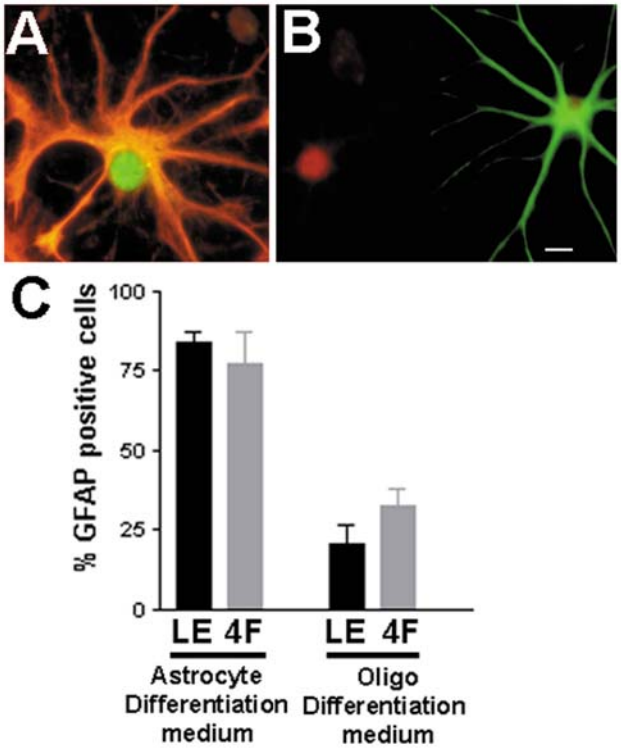

A
B
C
% GFAP positive cells
100
75
50
25
0
LE 4F
LE 4F
Astrocyte Differentiation medium
Oligo Differentiation medium

Figure 4

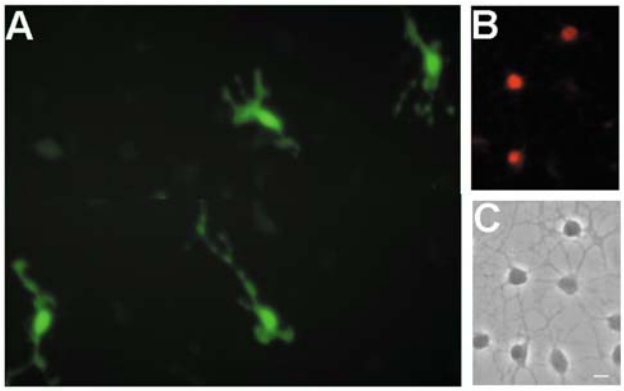
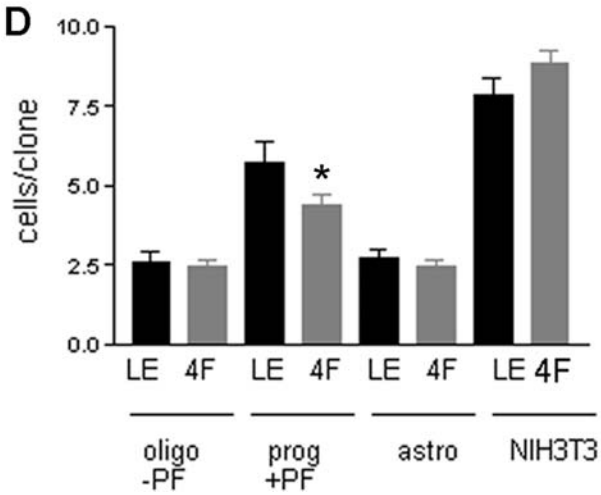

A
B
C

Figure 5


D
cells/clone
10.0
7.5
5.0
2.5
0.0
*
LE
4F
LE
4F
LE
4F
LE
4F
oligo
-PF
prog
+PF
astro
NIH3T3

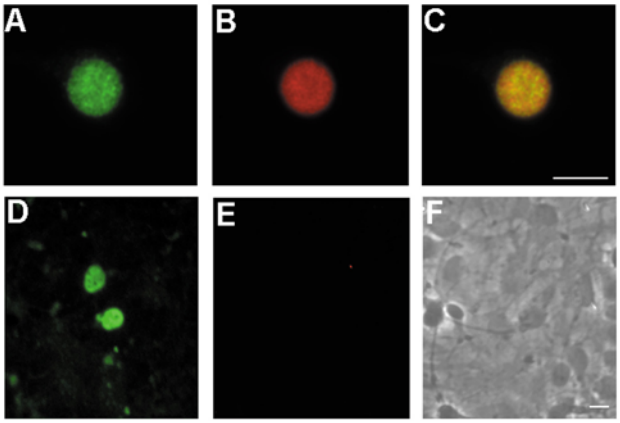


Figure 6

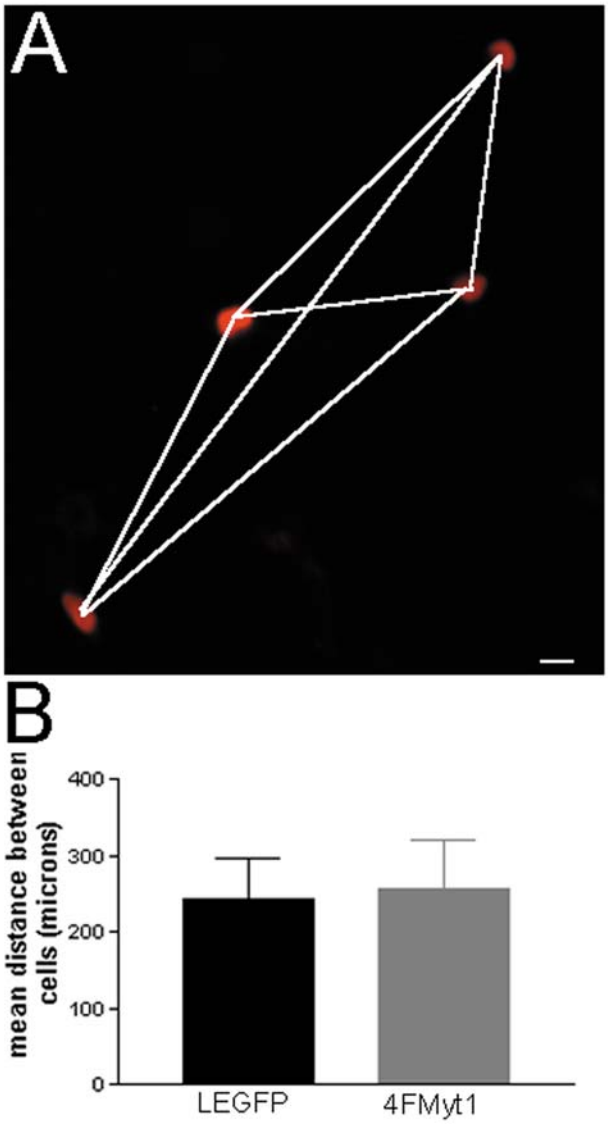

A
B
mean distance between cells (microns)
400
300
200
100
0
LEGFP
4FMyt1

Figure 7

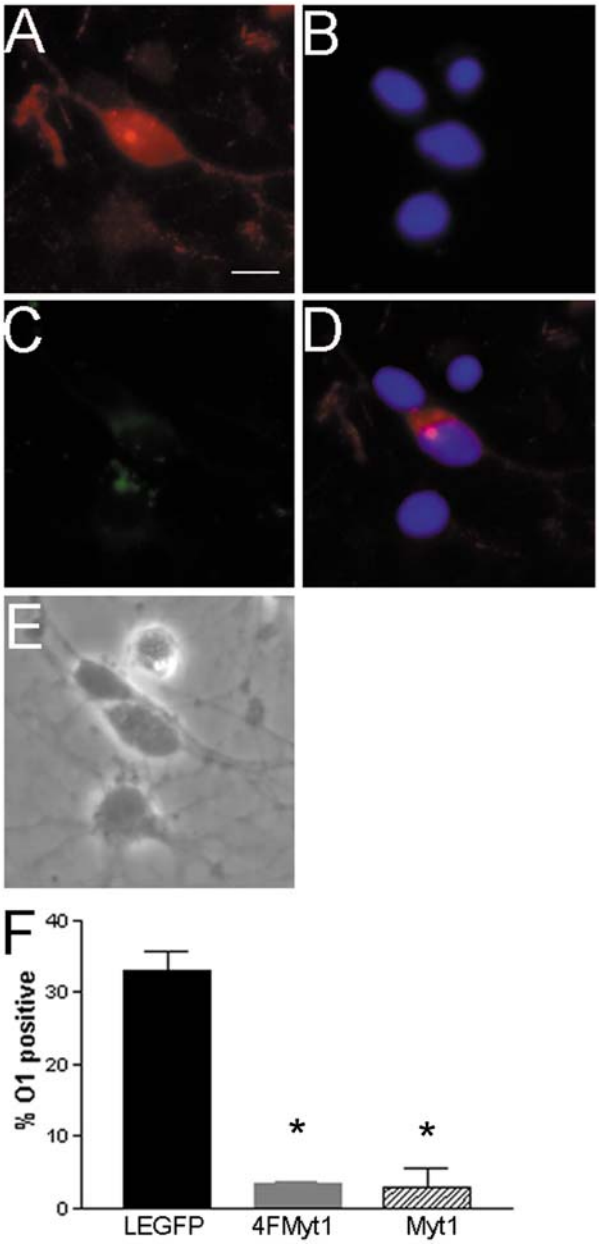


Figure 8

Discussion

In this study, we examined potential mechanisms regulating different aspects of oligodendrocyte differentiation and gene expression. We studied the contribution of gene and protein nuclear organization to the establishment and/or maintenance of terminally differentiated gene expression patterns, as well as the function of myelin transcription factor (Myt1) in the regulation of oligodendrocyte development.

Tissue-specific gene expression

To examine the role of nuclear organization and its contribution to tissue-specific gene expression, we used genomic in situ hybridization to localize the position of the *PLP* gene relative to nuclear proteins and a second myelin-specific gene, the myelin basic protein (MBP) gene. These experiments were performed in a primary cell culture model that undergoes developmentally regulated tissue-specific gene expression during terminal differentiation. The use of primary cells was important for these experiments because cell lines may not reproduce all aspects of differentiation induced gene regulation. Oligodendrocytes up-regulate a set of myelin-specific genes as they terminally differentiate. Many of these genes including the X-linked *PLP* gene and the autosomal *MBP* gene are expressed at similar developmental time points as oligodendrocytes terminally differentiate and both genes share similar regulatory factors such as the thyroid hormone receptor [39]. These observations led us to

hypothesize that the spatial colocalization of *PLP* and *MBP* genes might be an important mechanism for the coordinate regulation of these tissue-specific genes. The data collected in these experiments demonstrate that the *PLP* gene was not spatially associated with either *MBP* allele in progenitors where these genes are inactive, or after differentiation and up-regulation of *PLP* and *MBP* transcription in mature oligodendrocytes. Few studies have addressed the question of whether sets of tissue-specific genes exhibit spatial colocalization within the nucleus. One example that is available showed that immunoglobulin genes were differentially localized in the nucleus in two mature B-cell lines [52]. The data presented here along with the existing literature indicate that tissue-specific genes do not require spatial colocalization in order to coordinately regulate gene expression. However with this small sample size, it remains possible that other sets of genes may use this mechanism as means to facilitate coordinate gene regulation.

We also examined the localization of the *PLP* gene during oligodendrocyte differentiation. The literature contains a number of examples indicating that changes in the position of a gene within the nucleus may be an important regulatory mechanism [53, 54]. We hypothesized that the up-regulation of a tissue-specific gene like the *PLP* gene would be an excellent candidate to undergo developmental changes in nuclear localization for several reasons. The *PLP* gene is a single copy gene that requires very high levels of stable expression in differentiated cells. In addition, like many cells, oligodendrocytes contain heterochromatin at the nuclear periphery, so it was of interest to determine whether the *PLP* gene underwent a translocation from the nuclear

periphery to the nuclear interior during terminal differentiation and *PLP* gene activation. We show that the *PLP* gene is consistently associated with the nuclear periphery in oligodendrocyte progenitors and differentiated oligodendrocytes demonstrating that the *PLP* gene does not undergo any large-scale radial translocation away from the nuclear periphery during oligodendrocyte differentiation.

Nuclear lamins have been shown to bind chromatin [55], which indicates a potential mechanism for the apparent stable association of the *PLP* gene with the nuclear periphery. A chromatin attachment point near the *PLP* locus on the X chromosome could explain the association of the *PLP* gene with the nuclear periphery. Electron microscopy studies of oligodendrocytes have shown the presence of condensed heterochromatin at the nuclear periphery but there appears to be a non-uniform distribution of heterochromatin and euchromatin [22]. There are regions of euchromatin adjacent to the nuclear periphery where active genes could be positioned. Diamidino-2-phenylindole hydrochloride staining of the nucleus, which was used to define the nuclear periphery in these experiments, is unlikely to be able to detect small-scale chromosome movements. However, this method should be able to detect a large-scale radial translocation from the periphery to the nuclear interior within the nuclei of oligodendrocytes, which have a diameter of approximately 10 microns. The differential localization of the *PLP* and *MBP* genes and the stable association of the *PLP* gene with the nuclear periphery, demonstrate that the activation of a

tissue-specific gene does not require large-scale chromatin movement during oligodendrocyte differentiation and transcriptional activation.

The organization of nuclear proteins such as splicing factors have been shown to reorganize into larger clusters during cell differentiation [56]. We next addressed whether there were changes in the distribution of nuclear proteins during oligodendrocyte differentiation, which might contribute to terminally differentiated gene expression patterns. Splicing factors are organized into discrete nuclear domains called splicing factor compartments (SFCs). Our analysis allowed SFCs and the *PLP* gene to be compared at multiple stages of regulation of the *PLP* gene locus. Antibodies that detect the splicing factor SC35 immunolabel SFCs, and SFCs were detected adjacent to sites of transcriptionally active *PLP* genes in differentiated oligodendrocytes. There was not a marked association of SFCs in progenitors or astrocytes where the *PLP* gene is not expressed. There have been reports of embryonic expression of the *PLP* gene [57]. However using *PLP* mRNA in situ hybridization, we did not detect any marked *PLP* mRNA expression in oligodendrocyte progenitors in these culture conditions. Therefore, the presence of SFCs adjacent to the *PLP* gene only in mature oligodendrocytes is likely to be related to the high production of *PLP* transcripts.

One question that arises with this type of study is whether the association of active genes and SFCs are evidence of nuclear organization that facilitates efficient transcription and splicing. An alternative explanation is that the accumulations are merely a reflection of ongoing transcription and splicing [58].

Our data clearly demonstrates that SFCs are not associated with the *PLP* gene when the *PLP* gene is transcriptionally inactive. However, it is not possible to determine whether activation of the *PLP* gene and high transcription rates require the splicing factor compartments or whether they are caused by transcription and splicing. Studies of splicing factor nuclear organization indicate that there are low levels of splicing factors throughout the entire nucleus [59]. Therefore, it is unlikely that the initial activation of a gene requires being positioned adjacent to a SFC for efficient transcription and splicing. It is more likely that the initial splicing factor accumulations are caused by the activation of the *PLP* gene, and that ongoing transcription and splicing are subsequently made more efficient by the formation of SFCs adjacent to the site of transcription.

Splicing factors can be recruited to sites of transcription by the C-terminal domain of RNA polymerase II [36]. In addition, recent work indicates that the nucleus is an extremely dynamic environment with many nuclear proteins showing high rates of mobility throughout the nucleus [60]. SFC associations with transcription sites may be facilitated by two mechanisms: the rapid mobility of proteins diffusing throughout the nucleus and the interaction of splicing factors with high affinity binding sites, such as the C-terminal domain of RNA polymerase II at sites of active transcription. The protein-protein interactions could increase the residence time that splicing factors remain at sites of active transcription [61]. In oligodendrocytes, this process could result in the formation of SFCs adjacent to actively transcribed *PLP* genes, which may serve to facilitate

the high levels of transcription and splicing of the *PLP* locus in differentiated oligodendrocytes.

Myt1 Nuclear Organization

The distribution of nuclear proteins, such as transcription factors, can be organized into discrete nuclear domains and this has been taken as evidence for organizing principles within the nucleus. When oligodendrocyte progenitors are immunolabeled with a Myt1 antibody, a striking pattern of discrete nuclear domains can be observed [44]. An unresolved question is whether transcription factor domains are organized adjacent to their gene targets and represent active sites of transcription, or whether these accumulations represent storage sites or other undefined structures. The data for the DNA-binding protein Myt1 indicates that Myt1 domains are not associated with *PLP* gene activation. We examined preoligodendrocyte progenitors, oligodendrocyte progenitors, and differentiated oligodendrocytes at a stage when they were still expressing Myt1. There was an approximately 50% association of the *PLP* gene with Myt1 nuclear domains in each of these stages of oligodendrocyte development. There was also an approximately 50% rate of association of the transcriptionally silent interphotoreceptor retinoid binding protein gene with Myt1 nuclear domains. Taken together these data demonstrate that Myt1 nuclear domains are not sites of active transcription and may represent storage sites.

The number of transcription factor molecules required to bind to the promoter of a target gene to regulate transcription is likely to be relatively few,

which may explain why transcription factor domains do not correlate with active sites of transcription. Three binding sites have been identified in the *PLP* promoter for Myt1 [43]. If very few Myt1 proteins are required to regulate the *PLP* gene transcription, this would fall below the level of immunostaining detection capabilities. A higher resolution technique may be able to detect Myt1 at the *PLP* promoter during *PLP* activation. A recent study using a promoter array and GFP labeled transcription factors was able to show the recruitment of transcription factors to a gene undergoing transcriptional activation [62].

Transcription factor domains may still be functionally important even if detectable domains are not preferentially localized adjacent to target gene transcription sites. Localization of Myt1 to nuclear domains might serve as a mechanism to sequester and thereby regulate the concentration of available protein. The ability of a cell to regulate the amount of freely diffusing protein might be important for assuring that proteins only interact with the specific high affinity response elements and do not bind up essential limiting cofactors.

Myt1 Functional Study

The lack of association of Myt1 domains with *PLP* gene transcription sites failed to demonstrate a direct role for Myt1 in the regulation of *PLP* expression. However, the temporal and spatial expression pattern of Myt1 still indicated that Myt1 might play a role in regulating oligodendrocyte progenitor proliferation and differentiation. We examined Myt1 function in oligodendrocytes by expressing full-length Myt1 and the four zinc-finger DNA-binding domain of Myt1 (4FMyt1) in

oligodendrocyte progenitors. We show that retroviral mediated expression of 4FMyt1 has multiple inhibitory effects on oligodendrocyte function including proliferation and differentiation. In medium that promotes differentiation, expression of 4FMyt1 interfered with the differentiation of oligodendrocyte progenitors into mature oligodendrocytes as assessed by morphology, O1 immunoreactivity, and *PLP* mRNA expression. In addition to the effect of 4FMyt1 on differentiation, in the presence of growth factor mitogens expression of 4FMyt1 reduced the proliferation of progenitors.

In these experiments, we attempted to determine endogenous Myt1 function in a set of dominant negative experiments. We used a retroviral expression system to express the four zinc-finger DNA-binding domain of Myt1, which lacks the protein-protein interaction domain and the acidic putative transcriptional activation domain. Retroviruses are used extensively to study genes important in development because stable integration and expression allows one to follow the effects of expression over many cell divisions with clonal analysis. Our hypothesis was that expression of 4FMyt1 would compete with endogenous Myt1 for response elements in genes important for regulating oligodendrocyte function. The rationale for this approach is supported by examples in the literature, which take advantage of the functional domain organization of many proteins. In these examples the DNA-binding domain, in the absence of the transcriptional activation domain, can compete with the endogenous protein for response elements and reduce the function of the endogenous protein in a dominant negative manner [63, 64].

Expression of 4FMyt1 prevented oligodendrocytes from progressing to the mature oligodendrocyte stage of differentiation. The strongest evidence for this conclusion is the almost complete inhibition of 4FMyt1 infected cells that acquire O1 immunoreactivity. The O1 monoclonal antibody recognizes a galactocerebroside expressed on the cell surface of mature oligodendrocytes, and therefore can be used to identify the differentiated stage of oligodendrocyte development. The morphological and *PLP* mRNA expression data also support the conclusion that 4FMyt1 expression is preventing oligodendrocyte differentiation. We also show that the oligodendrocyte progenitors are not differentiating toward an alternative astrocytic cell fate using glial fibrillary acidic protein immunostaining. Together these results indicate that 4FMyt1 expression is maintaining cells at an immature stage of differentiation. In this study, the 4FMyt1 expression data is interpreted as evidence for a Myt1 role in promoting differentiation of oligodendrocyte progenitors. Consistent with these findings in rat oligodendrocytes, a study showed that overexpression of several dominant negative forms of a *Xenopus* version of Myt1, including the central domain and both sets of zinc-finger DNA-binding domains, inhibited neuronal differentiation [51]. We did not directly test the specificity of the 4FMyt1 effect on differentiation. A different primary cell type that does not express Myt1 could be used to test the specificity of 4FMyt1 on differentiation. Another possibility to test the specificity would be the creation of a non DNA-binding version of 4FMyt1. In these studies we used proliferation assays to test the specificity of the 4FMyt1 effect on oligodendrocyte function.

In addition to the 4FMyt1 effect on differentiation, we show that expression of 4FMyt1 inhibits proliferation. The decrease in proliferation measured by a reduction in clone size indicates that Myt1 may be regulating the expression of a cell cycle regulatory protein. In these proliferation experiments, we were able to directly test the specificity of the 4FMyt1 effect. Evidence for specificity of the 4FMyt1 effect is demonstrated by the lack of any effect in the proliferation experiments in astrocytes and NIH3T3 cells, which do not express endogenous Myt1. These data indicate that 4FMyt1 requires the presence of endogenous Myt1 and is specifically antagonizing Myt1 function.

Comparing the proliferation and differentiation data indicates a role for Myt1 in the transition from proliferating oligodendrocyte progenitors to mature oligodendrocytes. In the presence of platelet derived growth factor-AA (PDGF) and fibroblast growth factor-2 (FGF), there was a decrease in proliferation and no effect on differentiation indicating that 4FMyt1 is inhibiting proliferation without causing the cells to differentiate. In differentiation medium, there is no effect on proliferation, and a strong inhibitory effect on differentiation indicating that 4FMyt1 may be slowing or arresting oligodendrocyte progenitor development. In these experiments, we examined oligodendrocyte progenitor responses at two extremes of proliferation and differentiation signals. The growth factors PDGF and FGF are present in the proliferation medium at 10 ng/ml. In the differentiation medium there are very low levels of PDGF and FGF and high concentrations of T3 thyroid hormone and insulin in the medium. In vivo, oligodendrocytes are more likely responding to differing concentrations of PDGF,

FGF, and differentiation signals in combination with many other cell-signaling molecules. In order to better understand the function of Myt1, in vivo experiments which accurately reflect all the environmental signals that oligodendrocytes progenitors respond to should be tested (see future directions).

Full-length Myt1 Expression

To further characterize Myt1 function, transient transfections were performed to obtain full-length Myt1 expression data. Transfections were chosen because we were unable to generate enough full-length Myt1 virions. The distance from the 5' long terminal repeat (LTR) to the 3' LTR is an important limiting factor for the size of insert that can be used with a retroviral expression system. The upper limit is considered to be approximately 9kb [65]. The full-length Myt1 expression vector LTR to LTR size is 8.6 kb, which is near the limit and is likely reducing the titer. The overall size of the expression vector can also negatively impact retroviral titers. We observed a decrease in transfection efficiency as the vector size increased to 10 kb, which would decrease the number of packaging cells generating virus and lower the titer. However, another vector with a similar insert size did generate low titer virions in our hands, indicating additional problems with the full-length Myt1 retroviral expression vector.

Additional problems possibly caused by the repeat structure in Myt1 may be interfering with the retroviral expression system. Myt1 contains a 150 bp repeat in the N-terminus, which consists of GAA and GAG repeats encoding for

the acidic domain of Myt1. At the DNA level GAA repeats are reported to form an unusual helix structure with non-Watson/Crick base pairing [66]. We observed difficulty in sequencing through this repeat sequence, and needed to sequence the opposite strand and reposition the sequencing primer in order to get a sequencing read through this region. This indicates that the repeat sequence may affect polymerase processivity through the repeat, which could be interfering with transcription of the Myt1 sequence.

In addition, GAA repeats are associated with the disease Friedreich's ataxia. The mechanism of disease is unknown, but it has been suggested that the GAA repeats could bind RNA binding proteins leading to a global disruption of RNA splicing [66]. Evidence for this hypothesis is provided by the binding of GAA repeats to the RNA-binding protein Tra2 [67]. These findings could further explain why the full-length Myt1 sequence did not package efficiently. RNA-binding proteins may be binding to the GAA repeats in the Myt1 sequence and interfering with packaging of the retroviral transcripts and the viral structural proteins.

The subcellular localization of transfected full-length Myt1 in oligodendrocytes was predominantly cytoplasmic with a lower level of nuclear signal in most oligodendrocytes examined. This observation contrasts with the nuclear localization of 4FMyt1, which was nearly always found exclusively localized to the nucleus. Several weak consensus nuclear localization motifs are found within the 4FMyt1 sequence consisting of a hexapeptide containing three lysines and an adjacent proline amino acid [68]. The cytoplasmic localization of

Myt1 was surprising considering the full-length sequence contains the same nuclear localization sequences. In transfection experiments of Eco293, Cos cells, HeLa cells, primary astrocytes, primary neurons, and oligodendrocytes, we observed a predominately cytoplasmic localization of transfected full-length Myt1. In many cells, a major cytoplasmic aggregation of Myt1 could be observed. Endogenous Myt1 is found in discrete nuclear domains in the 0.2-1.0 micron size range. These observations indicate that Myt1 could be sensitive to overexpression. At the high levels of expression achieved with the CMV promoter in these studies, Myt1 may be saturating the nucleus and the excess may be exported to the cytoplasm. Myt1 contains a sequence very similar to the reported leucine-rich consensus nuclear export sequence LXXXLXXXLXL/I, which is found outside the 4FMyt1 sequence [69]. Excess Myt1 may be exported via the nuclear export domain, while 4FMyt1 is retained in the nucleus because it is lacking the nuclear export sequence. These results indicate that the levels of Myt1 in the nucleus may be regulated. The presence of consensus phosphorylation sites for protein kinase C indicates a potential mechanism for this regulatory process.

There is also the possibility that overexpression of Myt1 is saturating nuclear import leading to cytoplasmic accumulations. The expression level in transfections is more variable than with retroviral infections, and many transfected cells have high levels of expression. In transfection experiments, we did sometimes observe nuclear localization with cytoplasmic accumulations of 4FMyt1 in cells with very high levels of expression. This observation indicates

that at high levels of expression, 4FMyt1 may exceed the capacity of the nuclear import machinery. These observations also indicate that the tendency of Myt1 to aggregate into cytoplasmic domains may be mediated by the zinc-finger domains since 4FMyt1 alone also could be found in cytoplasmic aggregations. When we compared the nuclear localization of endogenous Myt1 to 4FMyt1 in a double immunostain, we observed a partial colocalization of 4FMyt1 with endogenous Myt1 domains. Taken together, these results indicate that the zinc-fingers of 4FMyt1 may be able to mediate the localization and/or association with endogenous Myt1.

In the transient transfection experiments, 4FMyt1 also inhibited differentiation of oligodendrocyte progenitors supporting the retroviral expression results. Surprisingly, transfected full-length Myt1 also inhibited differentiation of oligodendrocyte progenitors. One possible explanation for the similar effects of Myt1 and 4FMyt1 is the availability of a limiting cofactor. This effect has been reported to occur with overexpression of NCoR, a corepressor which binds the retinoic acid receptor and recruits mSin3 leading to transcriptional repression [70]. Deletion mutants lacking an mSin3 interaction domain, derepressed the reporter gene as predicted for the dominant negative NCoR in these experiments. However, overexpression of full-length NCoR also derepressed the reporter gene expression turning a corepressor into a transcriptional activator. The interpretation of this result was that another cofactor necessary for repression was limiting and the overexpression of NCoR was titrating this factor out leading to transcriptional activation [70]. Evidence for this hypothesis was

provided with a dose dependent transcriptional activation with increasing amounts of NCoR. This mechanism could explain the similar 4FMyt1 and full-length Myt1 effects on differentiation. Overexpression of Myt1 may sequester a limiting cofactor required for endogenous Myt1 function, while 4FMyt1 likely competes for response element binding with endogenous Myt1.

The dual functions of Myt1 in proliferation and differentiation seem contradictory. How could a protein be important for promoting both proliferation and differentiation, which must involve very different sets of genes? These results could be explained by the differential expression or regulation of Myt1 cofactors. There are numerous examples of transcription factors that are expressed in progenitors and not found in oligodendrocytes [39]. Since gene regulation requires the presence of cell type-specific and cell stage-specific transcription factors to regulate gene expression, the complement of transcription factors present at the different stages in oligodendrocyte development might account for the different effects of 4FMyt1 on both proliferation and differentiation. A second possibility is the regulation of the intracellular localization of Myt1 interaction partners. Id2 is a transcriptional repressor involved with preventing cell differentiation. A recent report demonstrated that the withdrawal of the growth factor PDGF can shift the localization of Id2 from the nucleus to the cytoplasm [71]. The sequestering of proteins in the cytoplasm could lead to different Myt1 functions depending on the factors that are present in the nucleus, and whether they could directly or indirectly regulate Myt1 interaction partners.

Future Directions

Important future experiments toward fully understanding the function of Myt1 in neural cell development include the identification of target genes of Myt1 regulation. In addition, the identification of interaction partners of Myt1 may help determine how Myt1 regulates gene expression and controls oligodendrocyte proliferation and differentiation. It would also be important to express Myt1 in an expression vector with a weaker promoter than CMV since Myt1 may be sensitive to overexpression. Additionally, a GFP-tagged Myt1 could be cloned into an inducible expression vector that could be turned on and off to observe Myt1 trafficking both between the cytoplasm and nucleus and within the nucleus to nuclear domains. Defining the mechanisms of nuclear import and export of Myt1 would also be an important step toward understanding how Myt1 is regulated and may lead to a more effective method for full-length Myt1 expression studies. An interesting set of experiments would be to inhibit nuclear export with leptomycin B, which inactivates CRM1-dependent nuclear export [72], and determine if Myt1 is retained in the nucleus. Additionally, the critical leucine residues in the consensus export sequence could be mutated to eliminate binding of CRM1, preventing nuclear export [69]. This Myt1 mutant could be used to study whether blocking Myt1 nuclear export has any functional consequences on oligodendrocyte function. The identification of signaling pathways that regulate Myt1 function post-translationally may also help determine how Myt1 regulates the transition from progenitors to differentiated oligodendrocytes. Protein kinase C has been implicated in the inhibition of

oligodendrocyte differentiation [73], and Myt1 contains multiple protein kinase C consensus phosphorylation sites indicating a potential link between signal transduction pathways and transcriptional control.

Finally, an in vivo analysis of Myt1 function by gene targeting would allow assessing the consequences of the complete absence of Myt1 expression on mouse CNS development. A less time consuming approach would be the use of a retroviral expression system for the delivery of Myt1 and Myt1 mutants into developing animals. This approach has the potential to identify the fates of cells that have reduced Myt1 function, and to determine the effects of reduced Myt1 function on the developing CNS, in the context of an otherwise normal CNS environment.

Conclusions

In summary, in this study we examined potential mechanisms regulating different aspects of oligodendrocyte differentiation. We examined the contribution of gene and protein localization to the establishment and/or maintenance of terminally differentiated gene expression patterns, and we studied the role of Myt1 in the regulation of oligodendrocyte proliferation and differentiation. These data support a nuclear organization model in which nuclear proteins and genes exhibit specific patterns of distribution within nuclei, and activation of tissue-specific genes is associated with changes in nuclear protein distribution. These data also indicate that Myt1 may regulate

oligodendrocyte lineage development at the transition between proliferation of oligodendrocyte progenitors and terminal differentiation.

These studies demonstrate the importance of nuclear organization to the regulation of oligodendrocyte progenitor differentiation, and the establishment of tissue-specific gene expression patterns. Additionally, this work is important toward understanding the role of Myt1 in oligodendrocyte development. A basic understanding of the mechanisms that underlie the regulation of the balance between proliferation and differentiation may be important in the development of future treatments for demyelinating and genetic diseases that affect oligodendrocytes and the myelin sheath.

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Appendix A

Abbreviations

APL-acute promyelocytic leukemia

CBP-CREB binding protein

CNS-central nervous system

EGFP-enhanced green fluorescent protein

FGF-basic fibroblast growth factor-2

LTR-long terminal repeat

MBP-myelin basic protein,

Myt1-myelin transcription factor 1

PGDF-platelet derived growth factor-AA

PLP-proteolipid protein

PML-promyelocytic protein

SFC-splicing factor compartment

4FMyt1-four zinc-finger DNA binding domain of Myt1